Regulation and functional aspects of *Xenopus* Brachyury.

Thesis

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Regulation and Functional Aspects of *Xenopus* Brachyury

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A thesis submitted for the degree of Doctor of Philosophy

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For the Mother Principle of the Universe

She creates, She evolves, She cares, She dissolves.

Science is discovering Her play

With the heart pure, the mind clear, the attention steady

She will show us the way.

Where is the purpose, what are the rules?

Do we see the beauty? Do we try to improve?

Nature is around us, nature is within, nature is in every cell.

Nature made our brain, nature made our body.

What did make our mind?

ACKNOWLEDGEMENTS

My appreciation for the help with the work for this thesis goes out from my heart to many people.

First of all I have to thank my supervisor Jim Smith. Thank your for your trust, thank you for your patience. However, I thank you most for giving me freedom in finding my own way in science and never refusing your helping hand when I needed it. I came here to learn as much as possible. I learned a lot, not only in science, but also as a person – and believe it or not – much from you. How I will be able to apply it, only the future will show.

Thank you also to all my colleagues and former colleagues at the Division of Developmental Biology for all your advice and help. My special appreciation to Masa Tada and Elena Casey who were always accessible for technical advice and help whenever and wherever needed. A big thank you also to Branko Latinkic for numerous *Xbra* promoter constructs and help with cloning. Thank you to Frank Conlon for material and ideas of studding Brachyury protein. Thank you Niall Armes for all the interesting speculations and discussions. I know I was not always in the state of fully appreciating them, but I believe one of your ideas will make you famous one day. Thank you to Tas Khan, Margarida Trindade and Kathy Neal for being such wonderful fellow PhD students, talking with you always lifted me up. And thank you to my more recent 'lab-mates' Catherine Papin and Dunja Knapp for taking up with my proliferating space demands during the process of writing this thesis.

Thank you to Danny Huylebroeck and Jacques Remacle, my collaborators in the University of Leuven, Belgium, for the SIP1 mutations in the *Brachyury* promoter and thank you for your patience for me analysing them. Thank you to Richard Tyrell, Division of Protein Structure, NIMR, London for the collaboration in analysing MAP kinase phosphorylation of the Brachyury protein. Thank you also to Enrique Amaya for technical advice on the transgenic method.

Last but not least my heartfelt thanks go to my parents, for their never ceasing support, morally and financially. Thank you for giving me all the freedom and never asking anything in return.

ABSTRACT

Formation of the mesoderm is one of the earliest patterning events in the development of the embryo. *Brachyury* is restricted to the mesoderm and has become one of the most widely used markers for mesoderm formation. The *Xenopus* homologue *Xbra* has been shown to be both necessary and sufficient for formation of mesoderm.

Employing a nuclear transplantation technique to create transgenic *Xenopus* embryos I studied spatial and temporal regulation of a reporter driven by *Xbra* genomic sequences. The proximal 5' flanking region of the *Xbra* promoter was sufficient to confine expression of a reporter to the mesoderm during gastrulation. Deletion analysis and point mutations in putative transcription factor binding sites identified two repressor modules, which are necessary to restrict expression to the marginal zone during gastrulation. This part of the project suggests that the *Xbra* gene is not confined to the mesoderm by specific activation, but rather by repression in tissues where its activity is not required.

To learn more about the functional aspects of the Brachyury protein I created a series of constructs encoding Xbra protein with N-terminal and C-terminal deletions. Nuclear localisation of the protein requires two regions not related to known nuclear localisation sequences. Experiments investigating the dimerisation properties of the protein confirmed that the full-length Xbra protein, binds a palindromic consensus sequence as dimer. However, there was no evidence for Xbra dimers forming without binding to DNA. Finally, I investigated the possibility that the MAP kinase pathway regulates Xbra on a post-translational level. The experiments showed that Xbra is

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phosphorylated by MAP kinase *in vitro* in the linker domain, but not in the DNA binding domain. These results will form the basis for a more detailed study investigating the post-translational regulation of Xbra. The possibilities for future experiments will be discussed in this context.

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1. INTRODUCTION

The *Brachyury* (or T) mutation was first described in 1927, isolated in the course of X-ray mutagenesis experiments in mice (Dobrovolskaïa-Zavadskaïa, 1927). Brachyury means "short tail" and the name reflected a dominant trait affecting tail length. Heterozygous mice with a deletion of the *Brachyury* or T-locus have short tails, while homozygotes die *in utero* with a deficiency of mesodermal structures including notochord and somites posterior to the forelimb bud (Chesley, 1935). This mutant phenotype has fascinated researchers for more than 70 years, but it was not until 1990 that the Brachyury gene was identified by positional cloning (Herrmann *et al.*, 1990).

The expression of the Brachyury gene is consistent with the mutant phenotype. It is expressed in all newly formed mesoderm and is maintained in the undifferentiated notochord and the tailbud (Wilkinson *et al.*, 1990). Brachyury and its expression pattern have been conserved throughout the vertebrate phylum. Homologues have been cloned in diverse organisms like human (Edwards *et al.*, 1996), chick (Kispert *et al.*, 1995b; Knezevic *et al.*, 1997), frog (Smith *et al.*, 1991), zebrafish (Schulte-Merker *et al.*, 1994b), amphioxus (Holland *et al.*, 1995) and ascidians (Yasuo *et al.*, 1996; Yasuo and Satoh, 1994). The expression pattern of Brachyury and the mutant phenotype suggest that the gene plays an important role in posterior mesoderm and notochord formation. Thus, if we can understand how Brachyury is regulated and how it exerts its effects we should also get a better understanding of the formation and patterning of the mesoderm in the embryo.

Mesoderm formation has been most extensively studied and is probably best understood in the amphibian embryo. Therefore this organism provides an excellent basis to learn more about the regulation and function of Brachyury. In the remainder of this chapter I will review what is known about mesoderm formation in *Xenopus* with some comparison to the process in other vertebrates. Then, I will go into more detail about what is known about Brachyury and describe my approach to learn more about its regulation and function.

1.1. Formation of the mesoderm in the *Xenopus* embryo

The early *Xenopus* embryo

Embryonic development is a fascinating process. It starts with a single cell, the newly fertilised egg. The fertilised *Xenopus* egg has all the necessary resources to complete embryonic development to the swimming tadpole stage in a simple salt solution. The first 12 cell divisions happen synchronously without transcription from the genome. This means that until this stage only the RNA and proteins supplied by the mother and stored in the oocyte are the driving force of development. It is also this information supplied by the mother that is responsible for triggering the patterning events that follow the start of transcription at the mid-blastula transition (MBT) during the 13th cell division (Kirschner *et al.*, 1985). By the 12th cell division there are three morphologically distinguishable areas in the embryo – these are the animal pole compromised of small pigmented cells, the vegetal pole with large, yolky, unpigmented cells, and the marginal zone in the equatorial region (Figure 1.1A).

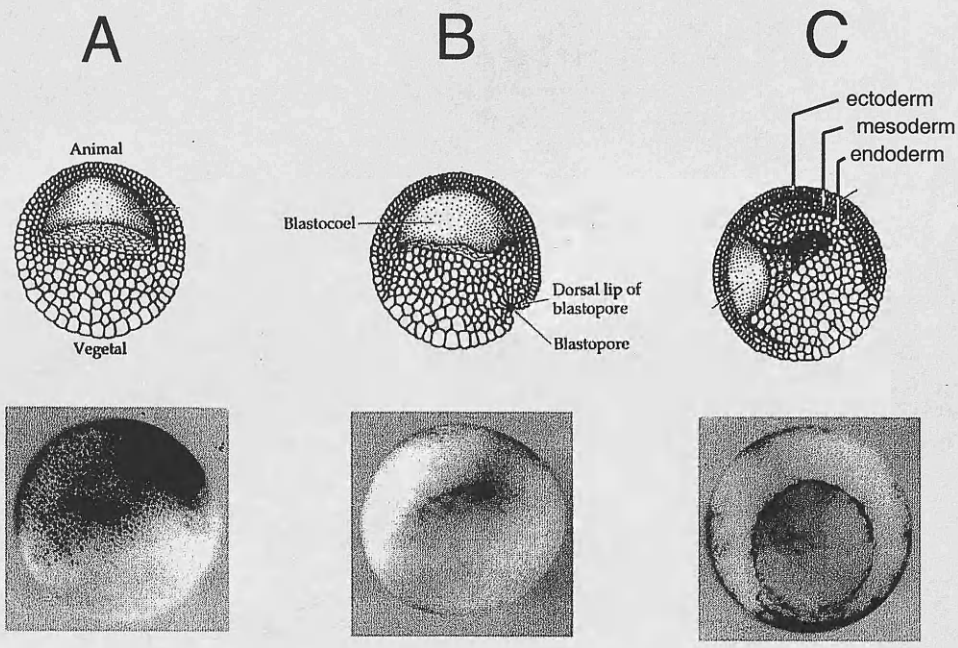


Fig.1.1. Cell movements in the gastrulating embryo. A. Before gastrulation the embryo is radial symmetrical, with the small, darkly pigmented animal pole cells on top and the large yolky, unpigmented cells below. **B.** At the beginning of gastrulation an invagination begins to form below the dorsal marginal zone and marginal zone cells are starting to migrate into the embryo. **C.** At mid gastrulation the invagination movement has spread form the dorsal to the ventral side, and the final distribution of the germ layers (ectoderm outside, endoderm inside and mesoderm in between) takes its form. Note that the cells that have started to move in on the dorsal side in B have moved along the dorsal side of the embryo towards the future anterior end, while the ventral cells are only just beginning to move in.

Mesoderm becomes apparent approximately four hours after MBT at the beginning of gastrulation, when cells deriving from the marginal zone start to involute inside the embryo, giving rise to the dorsal blastopore lip (Figure 1.1.B). This movement of cells involuting spreads from the dorsal side to the ventral side, until the blastopore is visible as a full circle (Fig. 1.1C). As a consequence of this movement also the vegetal cells are pulled inside the embryo and will give rise to endoderm.

The three germ layers are set up at the end of gastrulation. The animal pole cells above the equatorial region will form the ectoderm, the vegetal cells on the inside of the embryo form the endoderm, and the cells of the equatorial region form a layer between the ectoderm and the endoderm. Accordingly, the layer formed by the equatorial region is termed the 'mesoderm'.

The process of gastrulation always starts in a particular position in the embryo, opposite the site of sperm entry and just below the equatorial region. This positioning is important because it demarks the beginning of axis formation. The cells migrating from this position will move along the future dorsal side of the embryo, with the leading and furthest moving cells giving rise to anterior structures. The site of involution movement spreads from the dorsal side around the vegetal region to the ventral region, with the cells on the dorsal side involuting furthest and the cells on the ventral side involuting least (Fig. 1.1C).

While gastrulation causes the anatomical placement of the germ layers, their positional and identity cues are being prepared at a molecular level several hours before (reviewed in Harland and Gerhart, 1997). Although the *Xenopus* egg is radially symmetrical, it is polarised along its animal – vegetal axis, clearly visible by the difference in pigmentation. This polarisation will later be reflected in the positioning of the germ layers, with the animal hemisphere giving rise to ectoderm and mesoderm, and the vegetal hemisphere giving rise to endoderm. Several mRNAs have shown to be specifically localised to the vegetal pole, such as the TGF-ß member *Vg-1* (Weeks and Melton, 1987)

and the transcription factor *VegT* (Zhang and King, 1996) also known as *Xombi* (Lustig *et al.*, 1996), *antibodian* (Stennard *et al.*, 1996) and *Xbrat* (Horb and Thomsen, 1997). Both *Vg-1* and *VegT* are thought to have a role in the formation of the germ layers, as will be discussed later.

Mesoderm can be induced by the vegetal hemisphere

In 1969 Nieuwkoop published the results of an extensive series of experiments designed to understand the origin of the germ layers (Nieuwkoop, 1969). He dissected blastula stage embryos into different regions along the animal-vegetal axis, corresponding to animal pole, marginal zone and vegetal pole explants. Explants from the animal hemisphere developed into atypical epidermis and explants from the vegetal hemisphere developed into atypical endoderm, while explants from the marginal zone differentiated into structures that contained definitive mesodermal, endodermal and epidermal derivatives. However, the result that has fascinated researchers ever since, was that when explants from animal and vegetal hemisphere were combined, the resulting structures included derivatives from all three germ layers, including mesodermal tissues such as notochord, muscle, pronephros and blood. Interestingly most of the mesodermal tissues and even some of the endodermal derivatives had formed from the animal hemisphere tissue at the expense of ectodermal structures. Nieuwkoop's conclusion was that the vegetal explant induced the mesodermal structures to form from the animal pole explant, which then resulted in permissive signals for the ectoderm and endoderm to develop (Nieuwkoop, 1969).

When does the induction occur?

While Nieuwkoop's experiments conclusively showed that induction of mesoderm can occur, the questions still remain, of whether and how this process happens in the embryo and what is the signal? Several studies have addressed the timing of induction. Two aspects have to be taken into consideration. (i) When do the vegetal cells send the signal? (ii) When are the animal pole cells able to respond? Jones and Woodland (1987) addressed both these questions, by using heterochronous Nieuwkoop combinations. Their results suggested that ectoderm can respond to induction from stage 6 or 6.5 (32 – 128 cell) until stage 10.5, and that vegetal pole cells send an inductive signal over approximately the same time frame.

Another approach to determine the start of mesoderm induction is to ask when the marginal zone cells are actually specified to become mesoderm. This approach was first adopted by Nakamura and Takasaki, 1970), who identified stage 6.5 as the stage at which the mesoderm was determined. It was based on observations that isolated stage 6.5 equatorial zones, but not those of stage 6, showed subsequent mesodermal differentiation. The work was criticised, however, because of the difficulty in isolating equatorial zone cells without taking vegetal pole tissue (Nieuwkoop, 1973). More recently Ding *et al.*, 1998) repeated the experiment by labelling animal pole cells at the 8-cell stage, to ensure that isolated regions do not contain endoderm. They found that cells taken from the marginal animal pole region at the 16 cell stage, did not express the mesoderm markers *gsc*, *Xwnt8* or *Xbra* after MBT. However, if the same region was taken at the 128-cell stage, correct spatial and temporal localisation of the markers was observed (as analysed by RT-PCR at sibling stage 8.5 and 10.5). *Xbra* and *Xwnt8* were activated in explants from the ventral

marginal region, while *Xbra* and *gsc* were activated in explants from the dorsal marginal zone region.

These results seem to contrast with findings of Lemaire and Gurdon, 1994), who saw independent activation of *gsc* in dorsal marginal zone cells and *Xwnt8* in ventral marginal zone cells when embryonic cells were dispersed throughout development. This treatment would dilute any soluble inducing factors and suggests a direct activation of these genes by cytoplasmic determinants (Lemaire and Gurdon, 1994). However, in their experiments the marginal zone cells included also vegetal cells. Both *gsc* and *Xwnt8* are expressed not only in marginal zone cells that derive from the animal hemisphere and form mesoderm, but are also expressed in endodermal cells derived from the vegetal hemisphere (Lemaire and Gurdon, 1994; Zorn *et al.*, 1999). It was shown that cells from the animal hemisphere respond differently to maternal cytoplasmic determinants than cells from the vegetal hemisphere (Darras *et al.*, 1997), thus it is possible that mesoderm cells need an inductive signal to specifically activate *gsc* and *Xwnt8*, but vegetal cells do not.

Maternal or zygotic signals?

The strong implication of the above results is that the start of mesoderm induction must rely on maternal mRNA or protein, since transcription does not start until the mid-blastula stage at MBT (stage 8; approx. 4000 cells). However, they do not necessarily imply that all aspects of mesoderm formation and the formation of the other germ layers rely on maternal information. In particular, it is possible that a small maternal signal may initiate

a zygotic induction process or sensitise cells to further zygotic inductions (Harland and Gerhart, 1997).

Evidence for this view comes from experiments where blastula vegetal explants of different stages were combined with late blastula animal caps (St. 9), separated after one hour and analysed for mesodermal markers after a prolonged period of incubation (Wylie *et al.*, 1996). When the vegetal explants were used at the early blastula stage (stage 7) and separated before MBT the caps showed only weak activation of the ventral mesoderm marker *Xwnt8* (Christian *et al.*, 1991b). However, when the animal caps were combined with late blastula (stage 9) vegetal explants and separated after one hour, the animal caps showed strong activation of *Xwnt8* and *myoD*, a marker for dorsal mesoderm (Hopwood *et al.*, 1989). This argues that that a significant inductive signal takes place only after MBT and thus requires zygotic gene expression.

1.1.1. Mesoderm inducing factors

Ever since Nieuwkoop discovered that mesoderm can be induced from ectoderm by endoderm derived signals, much effort has been put into identifying the molecules responsible for this induction. Surprisingly many molecules were found that are able to convert ectoderm into mesoderm *in vitro* and the last decade has seen a race to establish which factor is actually responsible for mesoderm induction in the embryo. It turns out that it is probably a combination of factors that is responsible, but also that the formation of the germ layers is much more dynamic than a simple induction event.

Several members of the Transforming growth factor ß (TGFß) superfamily (Ring and Cho, 1999) and the Fibroblast growth factor (FGF) family (Slack *et al.*, 1996) are mesoderm inducers according to the original definition. Animal pole tissue cultured in dilute solutions of these mesoderm inducing factors differentiates into mesodermal cells types, while in absence of the factor it develops into atypical epidermis (Smith, 1987). The reason to search for secreted factors came from transfilter experiments, that showed that the animal hemisphere tissue does not need to be in physical contact with the vegetal tissue (Grunz and Tacke, 1986). However, to be a candidate for an *in vivo* inducer the factor should also be present in the embryo at the right time of development. Below I discuss the candidate mesoderm inducing factors and the evidence that each of them is an endogenous inducer.

i) Members of the TGFß superfamily

Members of TGFß family are secreted molecules. The precursor proteins form homodimers or heterodimers, which are subsequently cleaved to release a mature and active protein derived from the carboxy terminus (Vale *et al.*, 1990). The receptor for the TGF-ß family is a heterodimer of type I and type II receptors. Binding of the signalling molecule to a TGFß type II receptor induces the formation of a complex where the type II receptor phosphorylates and activates the type I receptor. The type I receptor then propagates the signal to downstream signal transducers, the receptor activated Smad proteins (Attisano *et al.*, 1994; Massague *et al.*, 1997). There are two distinct downstream pathways known, which are activated by different ligands. Activin, Vg-1, Nodal (Xnr) and TGFß signals are thought to be mediated by phosphorylation of Smad-2 or Smad-3 (Baker and Harland,

1996; Nomura and Li, 1998, reviewed in Whitman, 1998). Members of the bone morphogenic proteins (BMP) family are thought to signal via phosphorylation of Smad-1 or Smad5 (Hoodless *et al.*, 1996; Nishimura *et al.*, 1998). Upon activation the receptor activated Smads move to the nucleus (Baker and Harland, 1996; Baker and Harland, 1997). In addition they need also to form a complex with Smad4, which plays a crucial role in activating the transcription of TGFß responsive genes and stabilising the Smad/DNA complex (Chen *et al.*, 1997; Liu *et al.*, 1997).

The strongest evidence for a role for TGFß molecules in mesoderm induction comes from an experiment involving a truncated activin receptor (tActRII) that blocks signalling from all TGFß molecules tested (Hemmati-Brivanlou and Melton, 1992; Schulte-Merker *et al.*, 1994a; Hemmati Brivanlou and Thomsen, 1995), but does not affect signalling via FGF. Injection of high doses of RNA encoding tActRII into the fertilised egg severely disrupts mesoderm and endoderm formation (Hemmati-Brivanlou and Melton, 1992; Henry *et al.*, 1996). The phenotype can be rescued by co-injection of RNA encoding the wildtype receptor.

Activin

Activin is the best characterised mesoderm inducing factor. The protein is present in the early embryo, located in the yolk platelets of the vegetal hemisphere (Dohrmann *et al.*, 1993; Oda *et al.*, 1995), although it is not clear how it would signal from there to the animal hemisphere. Added in mature form to animal pole cells or injected as RNA into the embryo, it can induce a wide range of mesodermal and endodermal cell types in a dose dependent manner. Low concentrations induce ventral and posterior cell types, medium

concentrations induce muscle and notochord, while high concentrations activate endodermal cell types (Green *et al.*, 1992; Henry *et al.*, 1996). The only mesodermal cell type that cannot be induced by activin is blood, which originates from the most ventral mesoderm and needs high doses of BMP to differentiate (Xu *et al.*, 1999). It is, however, important to note that cells and tissues need an intact FGF signalling pathway to differentiate into mesoderm. (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994; Schulte-Merker *et al.*, 1994a).

There has been some doubt about the role of activin in the embryo, particularly because a targeted mutation in both activin subunits of the mouse gene had no effect on early development (Vassalli *et al.*, 1994; Matzuk *et al.*, 1995; Smith, 1995). However, a recent study used a more specific approach to inhibit activin in *Xenopus*. The extracellular domain of the activin type II receptor alone has a higher affinity to activin than to other TGFß members. Overexpression of this subunit does not inhibit signalling by Vg1 and Xnr1 but acts in a dominant negative fashion against induction by activin (Dyson and Gurdon, 1996). Embryos expressing this dominant negative form show a delay in mesoderm formation and develop with anterior truncations (Dyson and Gurdon, 1996). This results suggest that *Xenopus* embryos can form mesoderm without activin protein, but the delay in mesoderm formation is consistent with a role as sensitising signal for mesoderm induction that is then enhanced by zygotic signals.

Vg1

The *Vg1* gene encodes a maternal RNA that is localised to the vegetal region of the oocyte and the early embryo (Rebagliati *et al.*, 1985; Weeks and Melton, 1987). It is thus

expressed at the RNA level in the right place and at the right time to be an endogenous mesoderm inducer. However, although high levels of Vg1 protein accumulate as an unprocessed precursor in the embryo, no mature protein can be detected. Consistent with this, injection of *Vg1* RNA produces high levels of precursor, but no processed protein and consequently no mesoderm induction or developmental defects are observed (Yisraeli and Melton, 1988). In contrast, chimeric proteins consisting of the pro-regions of BMP2, BMP-4 or activin fused to the mature region of Vg1 are processed, rendering Vg1 biologically active (Dale *et al.*, 1993; Thomsen and Melton, 1993; Kessler and Melton, 1995). In these experiments the processed Vg1 protein behaves like activin to induce a range of mesodermal and endodermal tissues in a dose dependent manner.

Vg1 homologues have been cloned from zebrafish (Dohrmann *et al.*, 1996) and chick (Seleiro *et al.*, 1996). In both organisms the processed form of the protein is not detectable. Interestingly, when zebrafish Vg1 (zDVR-1) RNA is injected into *Xenopus*, the protein is processed and is a potent inducer of axial mesoderm. This finding suggests that Vg1 is subject to tight regulation at the level of post-translational processing (Tannahill and Melton, 1989; Thomsen and Melton, 1993).

Dominant inhibition of *Vg1* has proven to be a difficult task, perhaps because of the large amounts of unprocessed protein in oocyte and embryo. Joseph and Melton, 1998 used a point mutagenesis screen of *Vg1* to isolate mutants that are unable to signal and that specifically inhibit signalling by mature Vg1 in animal caps. The phenotype of the embryo is somewhat variable and difficult to interpret. In the most severe cases endoderm development is disrupted and axial structures are missing. However, in gastrula stages the

dorsal mesoderm markers, *Xnr3* and *chordin*, are slightly expanded and the ventral markers *Xvent-1*, *Xvent-2* and *Xwnt8* are slightly reduced. Expression of the pan-mesoderm marker *Xbra* is not affected. In contrast to this, injected embryos appear ventralised at later stages, with upregulation of the ventral marker *globin* and down regulation of *c-actin*, a marker for dorsal mesoderm. Although the phenotype would suggest a role for Vg1 in endoderm and dorsal mesoderm patterning rather than induction, it should be taken with caution until the mechanism of dominant interference is known. In particular the dominant negative form might interfere also with the recently identified Derriere, a zygotic expressed signalling molecule that has high homology with Vg1 (Sun *et al.*, 1999, see later).

Nodal and relatives

nodal was first recognised through its mutant phenotype in the mouse. Mice without functional nodal cannot initiate primitive streak formation and arrest at the gastrulation stage of development (Zhou *et al.*, 1993; Conlon *et al.*, 1994).

In the mouse embryo, nodal is initially expressed throughout the epiblast (the primitive ectoderm). Just before gastrulation it is expressed strongly in the prospective posterior region of the epiblast, thus marking the site of primitive streak formation. In addition, nodal has a domain of expression in the extra-embryonic endoderm. It is expressed initially throughout the visceral endoderm and becomes then restricted to the anterior region of this tissue (Varlet *et al.*, 1997). Varlet and colleagues constructed chimeric embryos in which a proportion of the embryonic tissues are wild-type for Nodal function and the extra-embryonic tissues are nodal -/-. In these embryos primitive streak

formation and thus mesoderm induction occurs, suggesting that the requirement for Nodal for these processes resides in the epiblast. Interestingly, these chimeras have anterior truncations, presumably due to a requirement for Nodal signalling from the visceral endoderm for anterior patterning. (Varlet *et al.*, 1997).

Four *nodal* related genes have been cloned in *Xenopus: Xnr1*, *Xnr2* (Jones *et al.*, 1995, *Xnr3* (Smith *et al.*, 1995b) and *Xnr4* (Joseph and Melton, 1997). Most interesting of these, with respect to mesoderm induction, are *Xnr1* and *Xnr2*. They are not expressed maternally, but transcripts are first detected uniformly throughout the vegetal hemisphere soon after MBT. At the early gastrula stage they become localised to the marginal zone, with stronger expression on the dorsal side. In the animal cap assay, both proteins induce endoderm and dorsal mesoderm (Jones *et al.*, 1995; Piccolo *et al.*, 1999), but not ventral mesoderm. The mouse mutant phenotype is also reflected in zebrafish embryos with double mutants of the nodal related genes *cyclops* (*cyc*) and *squint* (*sqt*). *cyc;sqt* double mutants lack all dorsal and anterior mesodermal derivatives and show severe disruption of endoderm development (Feldman *et al.*, 1998). The only mesoderm that forms is in the developing tail.

Dominant interference with nodal in *Xenopus* using injection of RNA encoding a dominant negative form of *Xnr2* (*cmXnr2*), which cannot be processed to an active form results in a delay of in onset of mesoderm markers and blastopore lip formation and a partial suppression of anterior endoderm markers. In addition the embryos show anterior truncations, possibly as a result of suppressed migration of the head mesendoderm (Osada and Wright, 1999). This phenotype is weaker than the phenotype of null mutants in

zebrafish and mouse, but is very similar to the specific interference with activin signalling (Dyson and Gurdon, 1996). This suggests that in *Xenopus* nodal signalling could play a role in relaying a maternal activin signal, but that a parallel pathway, possibly triggered by *VegT* (see below), helps to establish the germ layers in this organism.

Derrière

Derrière is a very recently identified member of the TGFß family and appears to be specifically required for the development of posterior mesoderm (Sun *et al.*, 1999). Its expression is exclusively zygotic, and overlaps nearly completely with the maternal T-box transcription factor VegT (see below). The RNA is localised to the endoderm and marginal zone at the late blastula stage, to the dorsal marginal zone at the beginning of gastrulation, and in the ventral posterior mesoderm at the end of gastrulation. It has the greatest homology to *Vg*-1 and is a potent inducer of endoderm and mesoderm markers. Induction of mesoderm by Derrière is dependent on FGF signalling. Overexpression on the dorsal side partially disrupts head formation, while ventral injection results in formation of a secondary axis including notochord.

Overexpression of a dominant negative form of Derrière, which cannot be processed into its active form (cm-Derrière), disrupts formation of axial and trunk mesoderm, while head development appears normal (Sun *et al.*, 1999). The phenotype is similar to injection of a dominant negative FGF receptor RNA (Amaya *et al.*, 1991) or ventral injection of RNA encoding a dominant negative version of *VegT* (Horb and Thomsen, 1997). The fact that VegT can induce *derrière* and Derrière can induce zygotic

expression of *VegT/Antipodean* (Stennard *et al.*, 1999) makes it likely that it has a role in an FGF dependent feedback loop important for the formation of posterior mesoderm.

BMP

Members of the Bone morphogenetic protein family (BMP), such as *BMP-2*, *BMP-4* and Bmp-7 are by themselves only very weak mesoderm inducers, as high doses are needed to induce ventral mesoderm in animal caps (Dale *et al.*, 1992; Jones *et al.*, 1992). All three have ubiquitous maternal expression, but do not show localised expression at the early blastula stage, arguing against a role as primary inducing factors (Fainsod *et al.*, 1994). *BMP-4* appears to be primarily involved in formation of ventral mesoderm. Its expression is strongly upregulated in the marginal zone at the beginning of gastrulation and extends far into the animal pole, but is absent from the most dorsal part of the embryo (Fainsod *et al.*, 1994; Schmidt *et al.*, 1995). *Xenopus* embryos injected with *BMP-4* mRNA are strongly ventralised (Dale *et al.*, 1992). However, ventralisation by BMP-4 is likely to act after the beginning of gastrulation, because initial blastopore lip formation occurs normally (Jones *et al.*, 1996).

An important role for BMP-4 in mesoderm formation is, however, suggested by the fact that most mice without functional BMP-4 arrest before gastrulation and do not form mesoderm (Winnier *et al.*, 1995). Inhibition of BMP signalling in *Xenopus* using a truncated BMP receptor or BMP binding molecules results in hyper-dorsalised embryos (Suzuki *et al.*, 1994). Thus BMPs seems to have an essential role in the formation of ventral mesoderm, but the experiments in mouse could suggest that they also have a permissive role in mesoderm induction.

ii) FGF-Family

Members of the FGF family are secreted molecules whose signal transducing receptors have constitutive tyrosine kinases activity. Ligand binding induces the formation of receptor homodimers and mutual phosphorylation (Schlessinger, 1988). Signal propagation involves the MAP kinase cascade (Umbhauer *et al.*, 1995; Gotoh *et al.*, 1995; LaBonne *et al.*, 1995). FGFs and activated components of the MAP kinase pathway are able to induce different kinds of mesoderm in animal pole tissues in a dose dependent manner. The response to FGF is different to that of activin in that thresholds are less defined and even the highest concentration of FGF cannot induce notochord or endodermal structures. (Green *et al.*, 1992; Green and Smith, 1990).

Several members of the FGF family are expressed during early embryonic development (reviewed in Slack *et al.*, 1996). *basic FGF* (*bFGF*), also known as *FGF-2*, is expressed maternally and then uniformly in all three germ layers (Kimelman *et al.*, 1988). *embryonic FGF* (*eFGF*), or *FGF-4*, has a maternal component, and its expression increases significantly during gastrulation when it becomes enriched in the dorsal marginal zone. As gastrulation proceeds, eFGF is expressed in a complete circle above the blastopore (Isaacs *et al.*, 1992). *Xenopus* FGF-3 (Tannahill *et al.*, 1992), *Xenopus FGF-8* (Christen and Slack, 1997) and *Xenopus FGF-9* (Song and Slack, 1996) are also expressed in the marginal zone, but are less well characterised than *bFGF* and *eFGF*.

As with the TGFß family, the most compelling evidence for a role for FGFs in mesoderm formation stems from dominant negative experiments. Expression of a truncated FGF receptor disrupts all trunk and tail mesoderm formation. In contrast, head formations occurs normally (Amaya *et al.*, 1991; Amaya *et al.*, 1993). In animal caps a truncated FGF receptor also disrupts most induction of mesoderm by activin (LaBonne and Whitman, 1994; Cornell and Kimelman, 1994; Schulte-Merker *et al.*, 1994a), whereas FGF is even more effective in mesoderm induction when TGFß signalling is inhibited (Hemmati-Brivanlou and Melton, 1992).

In the embryo, however, endogenous FGF does not rescue mesoderm induction in embryos when TGFß signalling is inhibited (Hemmati-Brivanlou and Melton, 1992). It was suggested that rather than being a natural inducer of mesoderm, that FGF is required in the maintenance of mesoderm. A role in maintaining trunk mesoderm was confirmed using transgenic frog embryos that express a truncated FGF receptor after MBT (Kroll and Amaya, 1996). While these embryos initiate expression of mesodermal markers in the late blastula stage, expression is not maintained, and embryos fail to form notochord and posterior mesoderm. As with embryos injected with mRNA encoding the truncated FGF receptor, head formation occurs normally (Kroll and Amaya, 1996).

An importance of FGF signalling in restricting the spatial borders of the marginal zone is suggested by following the observations. (i) Unlike activin, FGF induces mesodermal markers when added to or overexpressed in endodermal explants (Cornell *et al.*, 1995). (ii) Inhibition of FGF signalling along with inhibition of BMP signalling results in endoderm formation from animal explants (Sasai *et al.*, 1996). In addition, although

isolated animal hemispheres injected with RNA encoding bFGF form mesoderm in isolation, there is little effect of overexpression of bFGF on the intact embryo (Kimelman and Maas, 1992). This observation highlights the fact that mesoderm induction is not a one-way process, but rather the spatial distribution of the germ layers has to be actively maintained.

1.1.2. Role of transcription factors in mesoderm induction

The above section showed the possible involvement of secreted factors in mesoderm induction. However, recent evidence has also highlighted the importance of a transcription factor family in mesoderm formation, which can activate nearly all other mesodermal genes, when ectopically expressed in the animal hemisphere. This family is characterised by its DNA binding domain, the T-box, a relatively large domain of approximately 200 amino acids, and is therefore called the T-box family.

Brachyury

The founder member of the T-box family is *Brachyury*, the main subject of this thesis. The role of Brachyury in mesoderm formation will be discussed in more detail in Chapter 1.3. However, it is interesting to note here that it is the only T-box gene implicated in mesoderm formation that is solely expressed in the prospective mesoderm at the onset of gastrulation. Rather than being involved in the initiation of mesoderm induction, its expression appears to be a response to mesoderm induction. As will be discussed later, it might play an important role in defining the spatial distribution of the newly formed mesoderm and its ability to further differentiate.

Eomesodermin

Eomesodermin (*Eomes*) is a recently cloned T-box gene in *Xenopus* (Ryan *et al.*, 1996). It is expressed soon after MBT and reaches its maximum expression levels 1-2 hours before *Xbra*. In contrast to *Xbra,* its expression domain is wider and includes the goosecoid expressing anterior mesenendoderm and probably some prospective pharyngeal endoderm (Ryan *et al.*, 1996; Stennard *et al.*, 1999). Ectopic expression in the animal hemisphere activates most known mesoderm specific genes including *gsc*, *chordin*, *Xbra* and *Xwnt8* in a dose dependent manner, and additionally some genes such as *Mix.*1 which are more associated with endoderm formation. At the beginning of gastrulation *Eomes* expression is graded, with stronger expression on the dorsal side. Like *Xbra*, expression of *Eomes* is induced by several TGFß molecules and FGF without the need for protein synthesis (Ryan *et al.*, 1996). It is also induced by itself and by *VegT* (see below), but not by *Xbra* (Stennard *et al.*, 1996).

During gastrulation expression of *Eomes* becomes excluded from the most dorsal mesoderm and presumptive notochord domain, but persists in the most anterior mesoderm (Ryan *et al.*, 1996). The mouse homologue of *Eomes* (*mEomes*) is expressed in the primitive streak and the anterior visceral endoderm during gastrulation. (Ciruna and Rossant, 1999; Hancock *et al.*, 1999). The conserved expression pattern during gastrulation makes a role in mesoderm formation likely. However, further studies concerning its role in this process are needed.

VegT

VegT (Zhang and King, 1996), also known by the names *Antipodean* (Stennard *et al.*, 1996), *Brat* (Horb and Thomsen, 1997), and *Xombi* (Lustig *et al.*, 1996), encodes a maternal transcription factor that is specifically localised to the vegetal pole of the oocyte and the embryo before MBT. Zygotic expression of *VegT* starts soon after MBT in the dorsal marginal zone, and encompasses all the presumptive mesoderm at beginning of gastrulation*. During gastrulation, *VegT* becomes excluded from the presumptive notochord and persists in lateral and posterior mesoderm. Like *Eomes*, it can induce most mesodermal markers, including *Eomes* in a dose dependent fashion, but at a much lower concentration. Its expression is induced by TGFß and FGF in an immediate early fashion.

Depletion of the maternal component of *VegT* leads to a complete alteration of the germ layers (Zhang *et al.*, 1998). Endodermal fate is changed to mesoderm and ectoderm, such that no definite endoderm develops, but notochord and neural tube structures form from vegetal pole cells. The marginal zone stays on the outside of the embryo and forms ectoderm, while the animal hemisphere cells develop into a ventral cyst of pigmented cells. No head develops, but the anterior pole can be recognised by a cement gland (Zhang *et al.*, 1998). Analysis of molecular markers confirms that no endoderm forms but early mesodermal markers are expressed, albeit significantly delayed compared to control embryos. Highlighting the role of *VegT* in mesoderm induction, vegetal explants completely lose the ability to induce mesoderm in wildtype animal explants. The

* A recent report by Stennard *et al.*, (1999) has shown that the zygotic product is actually encoded by a different splice product of the *VegT* gene, resulting in a different N-terminus of the protein. For reasons of simplicity, however, I will refer to both products as VegT. No functional difference has yet been found.

phenotype can be rescued by injecting RNA encoding *VegT* into early cleavage stage embryos (Zhang *et al.*, 1998).

These results did not only highlight the important role of *VegT* in formation of the germ layers, they also gave an interesting twist to the view of mesoderm induction. VegT is a transcription factor and can presumably not act before MBT. Thus, any induction that might occur before MBT is not strong enough to be maintained without additional signals after MBT. Why then can marginal animal hemisphere cells isolated after the 64 cell stage develop into mesoderm by themselves (e.g. Ding *et al.*, 1998)? One explanation is that in the intact embryo the formation of the germ layers is a dynamic process that requires additional signals to actively maintain their identity.

The other intriguing result from the *VegT* depletion experiments is that mesoderm does eventually form, but this is not induced from the animal hemisphere but rather autonomously by the vegetal cells themselves. Several studies have shown that the activin-like TGFß molecules that can induce mesoderm in animal hemisphere tissues can also induce endoderm markers, albeit at higher concentrations (e.g. Gurdon *et al.*, 1996, Zhang *et al.*, 1998). Thus, *VegT* could be part of a positive feedback loop in the vegetal hemisphere to enhance TGFß signalling. Candidates for TGFß molecules induced by VegT are *Xnr-1*, *Xnr-2* and *derrière*, which are all, like *VegT*, initially expressed in the endoderm and later confined to the marginal zone (Jones *et al.*, 1995; Sun *et al.*, 1999).

1.2. Patterning the mesoderm

The last chapter dealt with the formation of mesoderm in the context of the other two germ layers. However, there is evidence that the mesoderm (as well as the embryo itself) is patterned along its dorsal/ventral axis from a very early time in development. This is, for example, reflected in the migration properties of the mesoderm. Migration of the marginal zone tissue inside the embryos always starts on the dorsal side with the formation of bottle cells, distinguishable by their concentration of pigment at their apical surface. This structure is called the dorsal lip. In addition, on a molecular level, several genes, such as *gsc*, *noggin*, *chordin* and *follistatin* occupy the region of the dorsal marginal zone, while others such as *BMP4*, *Xwnt8* or *myoD* are expressed only in the ventral and lateral marginal zone. Again others like *Xbra*, *eomes* and zygotic *VegT* are expressed in ventral, lateral and dorsal marginal zone. These changes are reflected later in the patterning of the mesoderm. The dorsal marginal zone tissue gives rise to notochord and muscle, while the ventral marginal zone gives rises to lateral plate, pronephros and blood cells. Thus to understand how mesoderm forms it is necessary to understand what specifies this difference between dorsal and ventral mesoderm. This will be discussed in the following section.

1.2.1. The Spemann organiser

The Spemann organiser represents the dorsal marginal zone tissue situated above the dorsal lip containing the newly induced dorsal mesoderm. The special properties of this tissue were discovered by Hans Spemann and Hilde Mangold (Spemann and Mangold, 1924). In their classic experiment a tissue fragment was taken from the dorsal lip of a

gastrulating embryo and grafted into the ventral side of a host of the same age. The host embryo developed a secondary axis with a good anterior to posterior organisation of a secondary neural plate, a notochord and in some cases, a gut. The difference in pigmentation between host and donor tissue made it clear that, although the grafted tissue differentiated into notochord and sometimes into floor plate and some somites, most of the secondary axis was induced from host tissue that otherwise would not have formed axial tissue (Spemann and Mangold, 1924). In honour of this initial experiment, the tissue was named after Spemann.

The experiment has been repeated in recent years, with cells marked with lineage tracer to document more precisely the contributions of graft and host to the secondary axis (Smith and Slack, 1983). These authors also performed the reverse experiment by placing the ventral marginal zone into the dorsal side of the embryo. While the dorsal marginal zone on the ventral side produced a secondary axis by converting the ventral tissue into dorsal axial tissue and keeping its normal dorsal fate, the ventral marginal zone on the dorsal side was converted into more dorsal tissue, such as somites. In the latter case most of the embryos looked apparently normal but had a twinned notochord, separated by the dorsalised donor tissue (Smith and Slack, 1983). This led the authors to propose a three signal model, whereby a pair of early inductive signals from the endoderm divides the early marginal zone into two distinct territories during the blastula stage: the dorsal (organiser) and the ventral mesoderm. The third signal, a dorsalising inductive signal from the organiser would then impose more dorsal and intermediate fates onto the ventral mesoderm in the gastrula stage (Dale *et al.*, 1985). A modified version of this model was suggested by Harland and Gerhart, 1997 and is shown in Fig. 1.2.

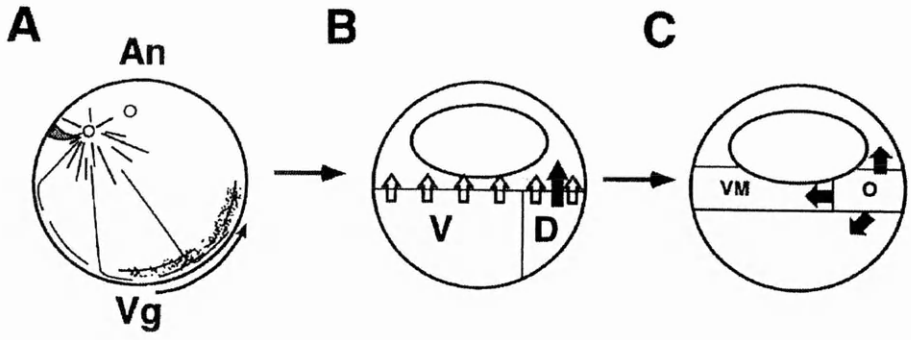


Fig. 1.2. Events of early amphibian development. A. Sperm entry causes redistribution of a vegetal cytoplasmic determinant to the future dorsal side. **B.** Two overlapping signals are present in the blastula embryo. A general mesoderm inducing signal acting over the whole area of the embryo from the vegetal pole towards the animal pole. Dorsal vegetal cells emit an additional signal that acts as dorsal modifier but does not have mesoderm inducing capacity. **C.** In the early gastrula stage the newly induced mesoderm is divided in two regions. The ventro-lateral marginal zone (VM) induced by the mesoderm inducing signal alone, and the dorsal organiser region (O). The organiser region releases a third signal toward the endoderm , mesoderm and ectoderm. On the endoderm this signal has anteriorising effect, on the mesoderm it has dorsalising effect and on the ectoderm it has neuralising effect. Taken from Harland and Gerhart (1997).

The initial division of the marginal zone into two sectors is also supported by other lines of evidence. The dorsal sector will develop by itself in isolation into dorsal mesoderm tissues, such as notochord and muscle (Dale and Slack, 1987). The rest of the marginal zone develops mostly only into the most ventral type of tissues, that is blood and mesenchyme (Dale and Slack, 1987). The dimensions of the late blastula organiser were determined by cutting embryo hemispheres along longitudinal meridians at low temperature and recombined them with naive ventral halves of the embryo. The conclusion was that at the late blastula stage the organiser occupies a 60° sector of the marginal zone centred on the point where the dorsal lip will form. If the organiser region is

absent the dorso-lateral marginal zone differentiates in the same way as the ventral half of the embryo, resembling a UV ventralised embryos (Stewart and Gerhart, 1990).

At the early gastrula stage the dimensions of the organiser widens to approximately 90° (Dale and Slack, 1987; Stewart and Gerhart, 1990), and there is considerable patterning in the marginal zone, such that a lateral muscle-forming region can be distinguished from a ventral region that forms blood and mesenchyme and the dorsal organiser region that forms notochord and muscle (Smith and Slack, 1983).

1.2.2. The dorsal determinant

The above section clearly depicts a dorsal/ventral asymmetry in the patterning of the newly formed mesoderm. How are these differences set up in the embryo?

Breaking the symmetry

The radial symmetry of the *Xenopus* egg is broken by sperm entry. The most visible sign is that the pigmentation of the embryo shifts approximately 30° in the direction of the sperm entry point during the first cell cycle, leaving the future ventral side more darkly pigmented than the dorsal side. This shift is accompanied by the movement of the vegetal cortex towards the future dorsal side (Vincent and Gerhart, 1986, Elinson, 1989). Although the cortex rotates by only about 30° relative to the core cytoplasm, small particles, membrane bound organelles and possibly informational molecules are transported in saltatory bursts for much greater distances, as much as 60-100° (Rowning *et al.*, 1997). The rotation is driven by the parallel alignment of microtubules oriented with their plus

pole towards the dorsal side (Houliston and Elinson, 1991). When cortical rotation is blocked by UV irradiation shortly after fertilisation, the embryo does not develop dorsal structures (Holwill *et al.*, 1987, Gerhart *et al.*, 1989). The result is a radially symmetrical embryo, that, at post gastrulation stages, consists of three concentric germ layers, the ectoderm, mesoderm and endoderm, all of which have ventral character. Interestingly, manually tipping the embryo 90° during the first cell cycle can result in a complete rescue of normal development (Scharf and Gerhart, 1980), and the extent and timing of tipping can determine the amount of rescue of dorsal structures (Gerhart *et al.*, 1984). This suggests that a dorsal determinant can be mechanically displaced from the vegetal pole into a region of the embryo where it can act to pattern the three germ layers.

The Nieuwkoop center

As a result of displacing the dorsal determinant a vegetal dorsalising center is created that is defined by its ability to induce dorsal mesoderm in cells of the animal hemisphere (Nieuwkoop, 1973). When the dorsal vegetal cells are transplanted to the same position on the ventral side of a normal embryo, a complete secondary axis can be induced (Gimlich and Gerhart, 1984), with the transplanted tissue only giving rise to endoderm. In honour of the pioneering work of Nieuwkoop this vegetal dorsalising center has been called the Nieuwkoop center (Gerhart *et al.*, 1989). Although the strongest Nieuwkoop center activity is found in the dorsal quadrant of the vegetal hemisphere, cells from the dorsal animal hemisphere have also inducing ability when transplanted to the ventral side (Kageura, 1990, Gallagher *et al.*, 1991). Cells of the dorsal animal hemisphere should secrete only a

dorsal modifying signal, as they are not able to induce mesoderm by themselves. These results again point to a separation between mesoderm induction and dorsalising activity.

By removal of cytoplasm including the cortex at different time points during the first cell cycle Kikkawa *et al.* (1996) established the movement of the dorsal determinant (Fig 2.3.). When cortical rotation is prevented by UV irradiation of fertilised eggs, movement of the dorsal determinant does not take place and the embryos fail to develop dorsal structures, although formation of the germ layers does occur (Gerhart *et al.*, 1989). The dorsal determinant instead stays localised to the vegetal pole (Darras *et al.*, 1997).

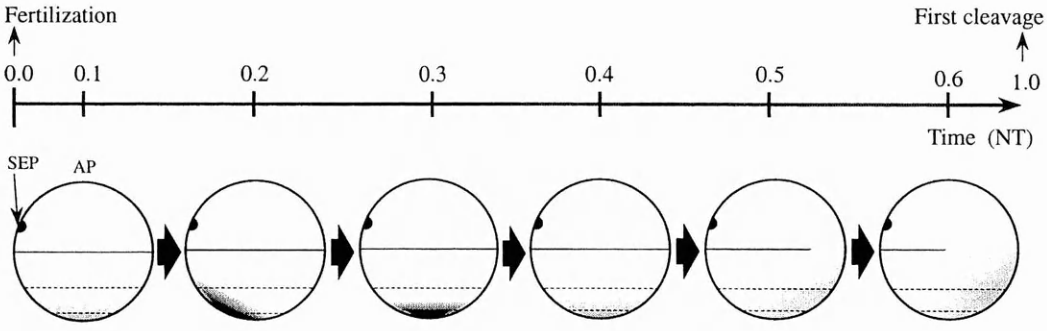


Fig.1.3. Illustration of presumed behaviour of dorsal determinants during the first cell cycle. The scale indicates relative times normalised with 0.0 as the time of fertilisation and 1.0 as the time of first cleavage. The darkness of the shaded portion indicates the concentration of localised dorsal determinants. Broken lines indicate latidudes 30° and 60° off the vegetal pole. AP animal pole; VP: vegetal pole; SEP: sperm entry point. Taken from Kikkawa *et al.* (1996).

1.2.3. The molecular nature of the dorsal determinant

One way of investigating the molecular nature of the dorsal determinant was to find molecules that are able to rescue axis formation in UV ventralised embryos. As with the

search for the mesoderm inducer, a surprisingly large number of molecules fit this description (reviewed in Heasman, 1997). There are three classes of molecules that show activity: i) Molecules that inhibit the BMP pathway (signalling via Smad-1), ii) TGFß factors that signal via the Smad-2 pathway, iii) Molecules that act positively in the Wnt signalling pathway. Additionally some transcription factors like *siamois* (*sia*) and *gooscoid* (*gsc*) have activity, but these are downstream of one or more of the above pathways.

i) BMP inhibitors

The three signal model (Smith and Slack, 1983) assumed that the ventral mesoderm is a ground state and that signals from the organiser can actively change this ground state into more dorsal fates. However, the model was challenged when it was found that injection of *BMP* RNA can not only induce ventral mesoderm, but also actively override the induction of dorsal mesoderm by activin as well as ventralise isolated dorsal marginal zone tissue (Dale *et al.*, 1992). Isolated dorsal marginal zone (DMZ) tissue, which includes the organiser, develops by itself mostly into notochord. However, when DMZ tissue is isolated from embryos injected with increasing concentrations of *BMP* RNA, their fate changes to muscle, pronephros and even blood forming tissue. In the same way, inhibition of BMP signalling by overexpressing a truncated BMP receptor changes the fate of ventral marginal zone (VMZ) explants in a concentration dependent manner to pronephros, muscle and notochord. In addition it induces a partial secondary axis when specifically directed to a ventral marginal blastomere in the whole embryo (Dosch *et al.*, 1997; Graff *et al.*, 1994). Thus, removing BMP-4 signals can unmask a dorsalising activity that is present throughout the underlying endoderm and marginal zone (Watabe *et al.*, 1995).

In light of these results it is perhaps not surprising that several of the molecules specifically produced in the organiser are inhibitors of the BMP signalling pathway. Noggin, Chordin and Follistatin are secreted proteins that directly bind BMP and thus compete with the BMP receptor (Piccolo *et al.*, 1996; Holley *et al.*, 1996; Iemura *et al.*, 1998). While BMP apparently does not diffuse very far, Noggin and Chordin can (Jones and Smith, 1998; Dosch *et al.*, 1997), and would be able to set up a functional BMP gradient of BMP, centred at the organiser.

However, BMP does not appear to exert its ventralising effect on the mesoderm before gastrulation (Jones *et al.*, 1996), and none of the BMP inhibiting molecules expressed in the organiser are expressed maternally. Thus, while this regulation of BMP signalling is undoubtedly an important mechanism for dorsal/ventral tissue specification, it does not act early enough in the embryo to be a candidate for the dorsal determinant.

ii) TGFß factors signalling via Smad2

Of the TGFß family members likely to signal via Smad-2, activin and Vg-1 are present maternally (Rebagliati and Dawid, 1993; Weeks and Melton, 1987) and both are able to induce ventral to dorsal mesoderm in a concentration dependent manner (Green *et al.*, 1992, Thomsen and Melton, 1993). Thus it would have been theoretically possible that an initial gradient of activin or mature Vg1 protein in the embryo directly induces ventral mesoderm at low concentrations and more dorsal mesoderm at higher concentrations (Gurdon *et al.*, 1994). However, there is no evidence for a difference in dorsal/ventral signalling of either of these proteins. When Watabe *et al.* (1995) used a reporter gene driven by multiple copies of an activin/Vg-1 response element of the goosecoid promoter

(which is mediated by Smad2, Labbe *et al.*, 1998), no difference was observed between the ventral and the dorsal side. The reporter is activated in all vegetal blastomeres, but not in the animal blastomeres. On the other hand if a *gsc* promoter fragment is used that also contains a Wnt response element, the reporter is activated to a 10 fold higher extent in the dorsal side than in the ventral side (Watabe *et al.*, 1995). A similar difference is seen when the Wnt response element is used without the activin/Vg-1 element, but with a much lower basal level. These results suggest that there is a dorsal/ventral difference in Wnt signalling, but Vg1/activin mediated Smad2 signalling is equally distributed throughout the vegetal pole.

Although overexpression of the molecules discussed in (i) and (ii) can rescue axis formation in UV ventralised embryos, they cannot induce a complete secondary axis when overexpressed on the ventral side of the embryo. Such secondary axis always lack head formation. Interestingly, this has a parallel in transplantation of tissue from the organiser. When the tissue is taken from a late blastula organiser, it has the ability to induce a complete secondary axis when transplanted to the ventral side. In contrast, when it is taken from the gastrula organiser it can induce a secondary notochord and somites, but not a secondary head (Gerhart, 1989). Thus, BMP inhibitors and molecules acting positively in the Smad2 pathway might play a role in mediating the signals form the late organiser, while the initial distinction between ventral and dorsal is set up by a pathway acting earlier in development.

iii) Molecules acting positively in the Wnt pathway

Several lines of evidence implicate the Wnt pathway (Fig. 2.4) in setting up dorsal/ventral differences during early embryonic development. As mentioned above, use of a promoter construct containing the Wnt-response element from the *gsc* promoter suggests that Wnt signalling is stronger on the dorsal side than on the ventral side of the embryo (Watabe *et al.*, 1995). In addition several members of the Wnt family, such as *Wnt8* (Smith and Harland, 1991) and Wnt1 (Christian *et al.*, 1991a) can induce a complete secondary axis when their RNA is injected into a ventral blastomere at the 32 cell stage. However, complete duplication of the embryonic axis can be induced not only by Wnts, but also by overexpression of positively acting downstream components of Wnt signalling such as Xfz8, Dsh, ß-Catenin, dominant negative *GSK3* and the transcription factors Twin and Siamois (Deardorff *et al.*, 1998; Dominguez *et al.*, 1995; Guger and Gumbiner, 1995; He *et al.*, 1995; Sokol *et al.*, 1995; Laurent *et al.*, 1997; Lemaire *et al.*, 1995).

Despite the ability of Wnts to induce a secondary axis, there is no Wnt gene known to be expressed at the right time and place for mediating primary axis formation. Moreover, injection of a dominant negative form of *Wnt8* can block secondary axis formation in response to Wnts, but does not block formation of the primary axis (Hoppler *et al.*, 1996). The same is true for a dominant negative form of Frizzled-8 (Deardorff *et al.*, 1998) and a dominant negative form of *dsh* (Sokol, 1996). By contrast, overexpression of wildtype *GSK-3*, dominant negative *Xtcf-3*, depletion of maternal *ß-catenin* by antisense RNA oligonucleotides and expression of a dominant negative form of Siamois, all of which lead to inhibition of the Wnt signalling pathway downstream of Dsh, lead to a disruption of the primary axis (Heasman *et al.*, 1994; Dominguez *et al.*, 1995; He *et al.*, 1995; Molenaar

et al., 1996; Fan and Sokol, 1997). These embryos have a ventralised phenotype that is very similar to the block of cortical rotation by UV irradiation. Thus if the dorsal determinant affects Wnt signalling it is likely to act downstream of *dsh*, presumably by local inhibition of GSK-3 (Moon and Kimelman, 1998).

Wnt signalling is mediated by accumulation of ß-catenin in the nucleus (Peifer *et al.*, 1994). Interestingly, such accumulation of ß-catenin can be seen on the dorsal side of the *Xenopus* embryo. By the 16 to 32 cell stage it is detectable in dorsal but not in ventral cells (Larabell *et al.*, 1997). At MBT the expression domain of nuclear ß-catenin encircles approximately one third of the equator and extends from the marginal zone into the vegetal and animal hemisphere with the center in the dorsal marginal zone (Schneider *et al.*, 1996). Ventralisation by UV treatment results in nuclear accumulation only in the most vegetal cells with no dorsal ventral difference (Schneider *et al.*, 1996). Thus the behaviour of ß-catenin accumulation very much reflects the spatial distribution of the dorsal determinant (Fig. 1.3).

Interestingly, neither induction of an axis by Vg-1 (via Smad2 signalling) or via noggin (a BMP inhibitor) affects the nuclear localisation of ß-catenin (Larabell *et al.*, 1997). Vg-1 and noggin, but not a dominant negative GSK-3, can rescue ventralisation by depletion of maternal ß-catenin RNA (Wylie *et al.*, 1996), suggesting that dorsalisation by Vg1 and noggin is a downstream event of Wnt-signalling by ß-catenin.

1.2.4. Positioning the organiser

Nuclear ß-Catenin acts as a transcriptional activator by forming a complex with the ubiquitously expressed transcription factor Lef-1/TCF-3 (Behrens *et al.*, 1996). Three downstream targets of ß-catenin that act during early *Xenopus* development are known to date: the closely related homeobox genes *twin* and *siamois* and the nodal related gene *Xnr3*.

All three have multiple TCF-3 binding sites in their promoters that confer dorsal specific expression (Fan *et al.*, 1998; Laurent *et al.*, 1997; McKendry *et al.*, 1997). All of them are expressed soon after MBT, before the zygotic expression of any other genes known to be involved in embryonic patterning. The expression domain of *siamois* and *twin* is centred on the dorsal vegetal hemisphere, but stretches deep into the vegetal pole (Laurent *et al.*, 1997; Lemaire *et al.*, 1995) and even into the animal hemisphere (Darras *et al.*, 1997). *Xnr3* is strongly expressed in the epithelial layer of the organiser tissue (Smith *et al.*, 1995a), but is also present in the dorsal vegetal domain (Darras *et al.*, 1997). *siamois* and *twin* can both reproduce all the effects expected from a dorsal determinant. When overexpressed in the animal hemisphere they do not induce mesoderm, but they strongly dorsalise mesoderm induced by either activin or FGF (Carnac *et al.*, 1996; Laurent *et al.*, 1997).

The only known direct downstream target gene of *siamois* and *twin* is the organiser specific gene *gsc*. *siamois,* as well as *twin,* binds to a homeodomain binding site in the Wnt response element of the *gsc* promoter (Laurent *et al.*, 1997). Interestingly, the *gsc* promoter has also an activin/Vg1 response element (Watabe *et al.*, 1995). This suggest

that a correct positioning of the organiser, requires a synergistic input from both, the mesoderm inducing pathway and the dorsal determinant. The transcriptional inputs that are necessary to position mesodermal genes will be discussed in more detail in Chapter 3. However, it can be expected that also other organiser specific genes show a similar regulation.

Another pathway that might help to position organiser specific gene expression is the BMP signalling pathway. Examples of target genes of this pathway are the homeobox genes *Vent1* and *Vent2/Xom* (Gawantka *et al.*, 1995; Ladher *et al.*, 1996; Onichtchouk *et al.*, 1996). Both are expressed in a domain excluding the organiser region (Gawantka *et al.*, 1995; Ladher *et al.*, 1996; Onichtchouk *et al.*, 1996). *Vent1* has been shown to negatively regulate the organiser specific gene *Xfkh1* (Friedle *et al.*, 1998), which is activated by the Vg1/activin pathway (Howell and Hill, 1997) and Vent2/Xom negatively regulates the organiser specific gene *gsc* (Trinade et al., in press).

Much has still to be learned about the process of mesoderm formation and patterning. The example of the *gsc* promoter shows that one way of gaining a better understanding of the mechanism of mesoderm formation is to study the promoters of mesoderm specific genes. The other important question is of how these mesodermal genes exert their effect. Most of the genes known to be expressed during mesoderm formation are either signalling molecules or transcription factors. Thus, to understand how they exert their effect we have to find the target genes that they regulate and what determines their specificity to these genes. Much work has been done in the recent years to address this question for the mesoderm specific gene *Brachyury*. In the next sub-chapter of the

introduction I will briefly review some of this research and mention the questions that are still open.

1.3. *Brachyury* and its role in mesoderm formation

Brachyury is an important gene in the analysis of mesoderm formation for several reasons.

First it is expressed in the presumptive mesoderm in mouse, frog, fish and chicken (Herrmann *et al.*, 1990; Wilkinson *et al.*, 1990; Smith *et al.*, 1991; Schulte-Merker *et al.*, 1992; Kispert *et al.*, 1995b; Knezevic *et al.*, 1997a). Initially expression occurs in all the newly formed primitive streak cells in the mouse, the germ ring in zebrafish and the marginal zone cells in *Xenopus*. As gastrulation proceeds, however, transcripts are lost from the newly involuted lateral and ventral mesoderm, but persist in the presumptive notochord tissue.

Second, lack of *Brachyury* function results in loss of the tissues in which the gene is expressed at the highest levels for the longest time. The mouse T mutant lacks mesoderm posterior to somite 7 and fails to form a properly differentiated notochord (Chesley, 1935; Herrmann, 1995). Similarly the zebrafish *no tail* mutant, which also lacks Brachyury function, fails to form posterior mesoderm and a notochord (Halpern *et al.*, 1993; Schulte-Merker *et al.*, 1994b). In *Xenopus*, overexpression of a dominant negative *Brachyury* construct produces an identical phenotype with loss of tail mesoderm and notochord (Conlon *et al.*, 1996).

Finally, misexpression of the *Xbra* gene in the prospective ectoderm of *Xenopus* embryos causes ectopic mesoderm to form in a dose dependent fashion (Cunliffe and Smith, 1992; O'Reilly *et al.*, 1995). Low levels of *Xbra* result in the formation of ventral cell types such as smooth muscle and mesenchyme, whereas higher levels result in the formation of skeletal muscle. Interestingly, even the highest levels of *Xbra* do not result in the formation of notochord. However, this tissue does form if *Xbra* is co-expressed with the secreted protein Noggin (Cunliffe and Smith, 1994), which inhibits BMP signalling (Zimmerman *et al.*, 1996), or with the transcription factor Pintallavis, encoded by a homologue of the mouse *HNF-3ß* gene (Ruiz i Altaba and Jessell, 1992; O'Reilly *et al.*, 1995). Neither *noggin* nor *pintallavis* alone can induce notochord when overexpressed in the prospective ectoderm.

The above observations mark *Brachyury* as a key gene in vertebrate mesoderm formation and it becomes therefore important to understand how its expression is specified to the prospective mesoderm and how it goes on to exert its effect in the formation of mesoderm.

Xbra is a direct response to mesoderm inducing factors

Putative mesoderm inducing factors were discussed in Chapter 1.1. One common feature of all these factors is that they turn on expression of *Xbra*, when ectopically expressed in the animal pole hemisphere. For activin and FGF it has been shown that this induction is direct (Smith *et al.*, 1991, Tada *et al.*, 1997a). Thus, *Xbra* is a direct response to mesoderm induction, and has in fact become a key marker for this process. Activin results in prolonged expression of *Xbra* only when present in a 'narrow window' of concentrations. Low levels of activin do not induce expression and if the dose of activin is too high, expression of *Xbra* is suppressed (Green *et al.*, 1992; Gurdon *et al.*, 1994; Gurdon *et al.*, 1995). It has been shown that the suppression of *Xbra* at high levels of activin is a secondary effect, possible mediated by genes like *gsc* and *Mix.1*, which are activated at high levels of activin (Latinkic and Smith, 1999a; Latinkic *et al.*, 1997; Artinger *et al.*, 1997; Lemaire *et al.*, 1998). This was suggested to be important for the localisation of *Xbra* to the marginal zone. In the vegetal pole concentration of an activin-type signal would be too high for the expression of *Xbra*, in the animal hemisphere it would be too low and in the marginal zone just right (Latinkic *et al.*, 1997). This model is probably too simple to explain the confined localisation to the marginal zone, but this will be discussed in more detail in Chapter 3.

Xbra expression is maintained by an indirect autocatalytic loop involving eFGF

The above section has addressed the question of how *Xbra* expression is induced in response to mesoderm induction, but the question remains of how expression is maintained. The importance of this point is shown by the expression pattern of *Xbra* in the

embryo. Initially expression occurs throughout the marginal zone, however, newly involuted cells rapidly lose *Xbra* transcripts, with exception of the most dorsal cells, destined to form the notochord.

Several experiments have shown that *Xbra* needs to be actively maintained after induction. If a marginal zone explant is cultured as an intact piece of tissue, expression of *Xbra* persists, however, if the same piece of tissue is cultured as dispersed cells *Xbra* expression declines rapidly, but this decline can be prevented when the cells are cultured in the presence of FGF (Isaacs *et al.*, 1994; Schulte-Merker and Smith, 1995). In addition, animal explants cultured in activin need a functional FGF pathway to maintain expression of *Xbra* (Schulte-Merker and Smith, 1995).

These results, together with the observations that ectopic expression of *Xbra* in the animal hemisphere causes activation of *eFGF*, (Isaacs *et al.*, 1994; Schulte-Merker and Smith, 1995), that *Xbra* and *eFGF* are co-expressed (Isaacs *et al.*, 1994), and that inhibition of FGF causes a down-regulation of *Xbra* expression (Amaya *et al.*, 1993; Kroll and Amaya, 1996), led to the idea that *Xbra* and *eFGF* might be components of an indirect autocatalytic loop in which Xbra induces expression of *eFGF* and eFGF maintains expression of *Xbra* (Fig. 1.4).

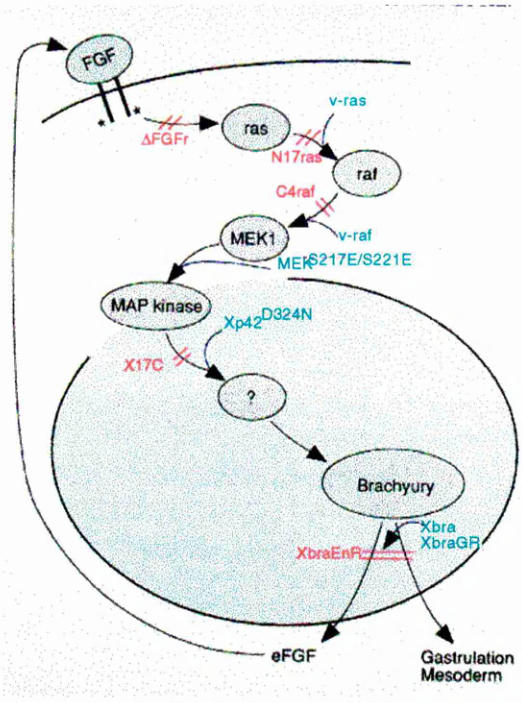


Fig. 1.4. The Xbra – eFGF autocatalytic loop and the reagents used to study it. Molecules indicated in red (ΔFGFr, N17ras, C4raf, X17C, Xbra-EnR) all inhibit a component of the loop; molecules in blue (v-ras, v-raf, MEK1$^{S217E/S221/E}$, Xp42^{D324N}, Xbra, Xbra-GR) represent the constitutively active forms of the MAP kinase pathway components or of Brachyury. The protein represented by "?" is a presumed target of MAP kinase activity. Taken from Smith *et al.*, 1997).

The importance of such a loop is shown by the fact that the ability of Xbra to induce mesoderm in the animal hemisphere is inhibited if FGF signalling is blocked in this tissue by a truncated FGF receptor (Schulte-Merker and Smith, 1995).

More recent evidence suggests that the FGF-Brachyury autoregulatory loop operates predominantly in the notochord. If Xbra function is blocked by overexpression of a dominant negative construct (see below), expression of the endogenous *Xbra* gene as well as *eFGF* is down-regulated in the notochord, but persists in the marginal zone (Conlon

et al., 1996; Casey *et al.*, 1998a). Similarly, expression of zebrafish *Brachyury*, *ntl*, declines in the notochord of *ntl* mutant embryos, but persists in the germ ring (Schulte-Merker *et al.*, 1994b). And in mouse embryos the initial primitive streak expression of Brachyury (T) is normal in T^{wis}/T^{wis} mutants, which lack Brachyury function (Herrmann, 1991). Thus, it seems unlikely that expression of Brachyury in the primitive streak, germring or marginal zone requires an autocatalytic loop, but at least in *Xenopus* and zebrafish the evidence supports the function of such a loop in the notochord.

Xbra is an Activator of Transcription

How does Brachyury exert its function in mesoderm formation? Soon after the gene was cloned (Herrmann *et al.*, 1990) it became clear that the amino-terminal half of the protein defines a conserved domain that became known as the T-box. Since then several other genes featuring this motive have been cloned and Brachyury became the founder of the T-box family, in which many of its members play an important role during embryonic development (Bollag *et al.*, 1994; Knezevic *et al.*, 1997b; Smith, 1999; Smith, 1997). Immunocytochemical analysis demonstrated that Brachyury is a nuclear protein (Schulte-Merker *et al.*, 1992; Cunliffe and Smith, 1994; Kispert and Herrmann, 1994) and binding site selection experiments established that the T-box binds a consensus 24 bp internally palindromic sequence AATTT(G/C)ACACCTAGGTGTGAAATT (Kispert and Hermann, 1993). Further studies showed that Brachyury is capable of activating transcription and activation domains were identified in mouse, *Xenopus* and zebrafish *Brachyury* (Kispert *et al.*, 1995a; Conlon *et al.*, 1996).

The conclusion that the main role of Brachyury is to activate transcription was tested by replacing the transcription activation domain of Xbra with the repressor domain of the Drosophila Engrailed protein (Han and Manley, 1993; Conlon *et al.*, 1996). Transient transfection experiments confirmed that this *Xbra-En^R* construct efficiently inhibits transcription activation caused by *Xbra* and when RNA encoding Xbra-En^R was microinjected into *Xenopus* or zebrafish embryos, this resulted in embryos lacking posterior mesoderm and, in many cases, notochord (Conlon *et al.*, 1996). These embryos therefore resembled mouse and zebrafish embryos lacking *Brachyury* function, leading to the conclusion that the main role of Brachyury is indeed to activate transcription.

Brachyury functions at a cellular level

Embryos developing without Brachyury function have posterior truncations and do not form a notochord, but what is the cause of this phenotype on a cellular level?
This question has been addressed in mouse using ES cell technology and in *Xenopus* making use of embryos and tissue manipulations on embryos injected with *Xbra-En^R*. In the mouse wildtype pre-implantation embryos were injected with T/T homozygous ES cells that carried a ubiquitously expressed LacZ marker, resulting in chimeric mice in which the distribution of individual cells lacking the T-gene could be observed (Wilson *et al.*, 1995). These experiments, and earlier studies without a LacZ marker (Rashbass *et al.*, 1991; Wilson *et al.*, 1993b), have shown that cells lacking Brachyury expression fail to migrate away from the primitive streak and thus accumulate at the posterior end of the embryo. Clumping of these cells caused the authors to speculate that this phenotype is due to abnormalities in the adhesion properties of the cells, rather than due to a defect in cell migration (Wilson *et al.*, 1995).

In *Xenopus* there are two types of cell movement involved in the process of gastrulation: migration of mainly anterior mesoderm cells and convergent extension, which causes elongation of the embryo along the anteroposterior axis (Gerhart and Keller, 1986). Both movements can be observed in mesoderm induced from prospective ectoderm by activin (Symes and Smith, 1987). Inhibition of Xbra function by Xbra-En^R inhibits the convergent extension movement of activin induced mesoderm, but does not affect cell adhesion and migration of these cells on fibronectin (Conlon and Smith, 1999). Conlon and Smith (1999) went on to show that cells lacking Xbra function, remaining in the posterior portion of the embryo are not converted into more anterior fates but undergo programmed cell death after the embryo has completed gastrulation.

In addition to its role in gastrulation movements, Brachyury also plays a role in differentiation. This has been conclusively shown for the notochord. Studies in mouse, zebrafish and *Xenopus* have shown that cells lacking Brachyury function are either not incorporated into the notochord or fail to differentiate into notochordal tissue (Wilson *et al.*, 1995; Halpern *et al.*, 1993; Conlon and Smith, 1999). This effect appears to be cell autonomous, because even single cells with a mutation in the *ntl* gene are not rescued in the notochord by their neighbours when transplanted into wildtype zebrafish embryos (Halpern *et al.*, 1993). In accordance with the suggestion of an autoregulatory loop between FGF and *Xbra*, animal hemisphere tissue cannot differentiate into mesoderm in response to FGF when the function of Xbra is inhibited by expression of Xbra-En^R (Conlon and Smith, 1999).

Downstream of Brachyury

If the role of Brachyury is to activate transcription, what are the genes whose expression it induces? The identification of direct targets of Xbra was helped by the use of a hormone-inducible version of Xbra, in which the open reading frame is fused to the ligand binding domain of the glucocorticoid receptor (Xbra-GR; Tada *et al.*, 1997). In absence of the glucocorticoid dexamethasone the Xbra-GR fusion protein is sequestered by the heat-shock apparatus of the cell and rendered nonfunctional. When dexamethasone is added, however, the fusion protein is liberated and is able to exert its effects. This allows specific activation of Xbra at a defined time point of development by dexamethasone. A similar approach had also been used to study MyoD and Otx2 (Gammill and Sive, 1997; Kolm and Sive, 1995). A direct target of Xbra-GR should be activated after addition of dexamethasone even in the presence of the protein synthesis inhibitor cycloheximide.

Two approaches to identify targets of Xbra have met with success: guesswork and a screen. One target gene that has been identified by guesswork is *eFGF*, which is involved in an indirect autoregulatory loop in which eFGF maintains expression of *Xbra* and Xbra maintains expression of *eFGF* (see above). Consistent with this idea, the expression pattern of these two genes are almost identical (Isaacs *et al.*, 1995), and Xbra-GR induces *eFGF* in the presence of cycloheximide and dexamethasone. Investigation of the eFGF promoter revealed the sequence TTTCACACCT (Casey *et al.*, 1998), which represents half of the previously identified Brachyury binding site (Kispert and Hermann, 1993). A similar 'half site' is also present in the 5' regulatory region of the human and mouse *FGF-4* gene, the mammalian homologue of *eFGF*. The observations that Xbra can bind to this sequence as a monomer and is able to activate transcription of a reporter gene placed downstream of the

half site (Casey *et al.*, 1998a), further added to the evidence that *eFGF* is a direct target of Xbra.

A screen to identify *Xbra* targets was carried out by Tada *et al.*, 1998), who used the hormone inducible Xbra-GR to construct a cDNA library enriched for genes rapidly activated by Xbra. Screening of this library yielded among other genes four cDNAs encoding homeobox proteins related to the Mix family. These genes were named Bix1-4 (for Brachyury induced homeobox containing genes). Interestingly, these genes were activated not only by Xbra but also by VegT. Like *Xbra*, they are expressed in the prospective mesoderm of the embryo, but like *VegT* they are also expressed in the vegetal hemisphere (Tada *et al.*, 1998). These observations suggested that they not only act downstream of *Xbra*, but also of *VegT*. Consistent with this suggestion overexpression of *Bix1* in animal caps causes formation of ventral mesoderm at low concentrations, but endoderm at high concentrations (Tada *et al.*, 1998), and the 5' regulatory region of *Bix4* contains the sequence CTTCACACCT, which binds VegT in addition to Xbra (Tada *et al.*, 1998). Further studies showed that *Bix4* can partly rescue endoderm formation in VegT depleted embryos, but cannot rescue the ability of the vegetal hemisphere to induce mesoderm (Casey *et al.*, 1999). Thus *Bix4* appears to mediate at least part of the function of *VegT* in the endoderm, and it will be interesting to wait for the characterisation of other genes from the screen that are involved in mediating the function of *Xbra* in the mesoderm.

It had been a surprise that Xbra could bind as a monomer to the half site. Previous work had suggested that Brachyury could not bind to a half site (Kispert and

Herrmann, 1993; Kispert *et al.*, 1995a) and that it functions as a dimer (Papapetrou *et al.*, 1999). Indeed the structure of the Brachyury DNA binding domain was solved as a dimer interacting with a palindromic sequence (Muller and Herrmann, 1997). The ability of T-box proteins to interact with a half site has also been observed by Carreira *et al.* (1998) who find that Tbx2 interacts with a single TCACAC core. In fact the only natural palindromic Brachyury binding site has been found in the notochord enhancer of As-T, the *Brachyury* homologue from the ascidian species *Halocynthia roretzi*. This site may act as an autoregulatory element (Takahashi *et al.*, 1999). No promoter of a *Brachyury* target acting in the notochord has yet been characterised, and it will be interesting to see if a palindromic target site will be a feature of such a promoter in higher vertebrates.

The search for Brachyury targets is still at an early stage. However, the above sections raise several questions that should be further elucidated: What determines the specificity of *Brachyury* in comparison to other T-box genes? Does Brachyury generally act as a monomer as suggested by the *Xbra* targets characterised so far, or can it also act as a dimer as suggested by *in vitro* studies involving binding to DNA (Kispert and Herrmann, 1993; Kispert *et al.*, 1995a; Papapetrou *et al.*, 1997; (Muller and Herrmann, 1997)? These and other questions concerning functional aspects of the Brachyury protein will be discussed in more detail in Chapter 4.

2. MATERIALS AND METHODS

Abbreviations

ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate
CTP	Cytidine 5'-triphosphate
$_dH_2O$	Distilled water
DTT	Dithiothreitol
EDTA	Ethylene-diamine-tetra-acetate
EGTA	Ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid
GTP	Guanidine 5'-triphosphate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
MMR	Marks Modified Ringers
MOPS	3-(N-morpholino)-propanesulfonic acid
NAM	Normal Amphibian Medium
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
SDS	Sodium lauryl sulfate
TTP	Thymidine 5'-triphosphate
UTP	Uridine 5'-triphosphate

Formulation of Frequently Used Solutions

PBS	137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4
TE	1mM EDTA, 10mM Tris.HCl pH 8.0
TAE	40mM Tris.actetate, 2mM $Na_2EDTA.2H_2O$ (pH 8.5)
TBE	89mM Tris-Base, 89mM Boric acid, 2mM EDTA pH 8.0
20X SSC	3 M NaCl, 0.3 M Na_3citrate.$2H_2O$, adjust pH to 7.0 with 1 M HCl
10X NAM-salts	110 M NaCl, 2 M KCl, 1 M $Ca(NO_3)_2$, 1 M $MgSO_4$, 0.1 M Na_2EDTA
10% NAM (500 ml)	5 ml 10X NAM salts, 10 ml 0.1M Na phosphate (pH 7.4), 2.5 ml 10 mg/ml gentamycin
75% NAM (500 ml)	37.5 ml 10X NAM salts, 10 ml 0.1M Na phosphate (pH 7.4), 5 ml 0.1M NaHCO3, 2.5 ml 10 mg/ml gentamycin
2% cysteine hydrochloride pH7.8-8.1 (200ml)	4.4 g L-cysteine hydrochloride monohydrate, 1.33 to 1.36 g NaOH pellets, fill up to 200 ml distilled water
10 X MEM salts	1 M MOPS, 20 mM EGTA. 10 mM $MgSO_4$
MEMFA (50 ml)	5 ml 10X MEM salts, 5 ml 37% formaldehyde
MMR	100mM NaCl, 2mM Kcl, 1mM MgCl2, 2mM CaCl2, 5 mM HEPES, pH7.5

Formulation of Frequently Used Bacterial Growth Media

LB (L-Broth)	1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 1% w/v NaCl
L-agar	L-Broth supplemented with 1.5% bacto-agar
2X TY	1.6% w/v bacto-tryptone, 1% w/v bacto-yeast extract, 0.5% w/v NaCl

2.1. Molecular Biology Techniques

Preparation and storage of competent bacteria

The DH5α strain of *Escherichia coli* was rendered competent for transformation by treatment with $CaCl_2$. A single colony was placed in 50 ml of L-broth and shaken at 37°C overnight. 10 ml of this culture was inoculated into 100 ml of P-medium (15.9 mM K_2PO_4, 6.3m M KH_2PO_4, 15 mM $(NH4)_2SO_4$, 10 mM $MgSO_4$, 1.8 mM $FeSO_4$, 1% casamino acids and 0.2% glucose) and cells were grown to an optical density of 0.3 at 600 nm. After washing in 100 ml of 10 mM NaCl at 4°C, cells were repelleted. Bacteria were resuspended in 100 ml of 50 mM $CaCl_2$ and incubated at 4°C for 15 minutes. Finally bacteria were pelleted and resuspended in 10 ml of 50 mM $CaCl_2$, 16% (v/v) glycerol, aliquoted and quickly frozen in dry ice before being stored at -80°C.

Plasmid transformation of competent bacteria

Frozen DH5-α competent bacteria were thawed on ice. Up to 100 ng of DNA was added to 100 ml of cells in Falcon 2059 tubes (Becton Dickinson). The bacterial cells were kept on ice for 15-30 minutes and then heat shocked at 42°C for 45 seconds followed by cooling on

ice for 30 minutes to 2 hours. After this period, 200 ml of LB was added and the mixture was incubated at 37°C for 15 minutes. Bacteria were then plated out onto LB plates containing 100 mg/ml ampicillin and placed in a 37°C incubator overnight. Alternatively, 100 to 1000 ng of DNA were added to 100 µl of pre-thawed DH5-α competent bacteria. The mixture was kept on ice for 20 minutes and then at room temperature for 10 minutes, 200 µl of LB was added and the mixture was plated out and incubated as above.

For defined plasmid recovery, 10 ml of competent DH5-α bacteria was added to 500 ng of plasmid DNA and placed at 37°C for 90 seconds, then 100 ml of L-broth was added to the mixture and this was plated out onto LB plates containing 100 mg/ml ampicillin. Plates were placed at 37°C overnight.

Small scale preparation of plasmid DNA

From a 3.5 ml overnight culture of transformed DH5-α bacteria in LB, 1.5 ml was transferred to a 1.5 ml microfuge tube and spun for 20 seconds. The supernatant was removed completely and the pellet resuspended in 300 µl of Resuspension Buffer (Qiagen; 10 mM EDTA, 50 mM Tris.HCl pH 8.0, 100µg/ml RNAse). 300 ml of Lysis Buffer (Qiagen; 0.2 M NaOH, 1% SDS) was added, mixed and left for 2 minutes at room temperature to allow alkaline lysis of the cells. Lysis solution was then neutralised by adding 300 ml of ice cold Neutralisation Buffer (Qiagen; 3 M KOAc pH 5.5) and mixing carefully by inverting the tube a few times followed by 10 minutes on ice. The tube was spun for 15 minutes at room temperature. 700 ml of the supernatant was transferred into a fresh microfuge tube and phenol/chlorophorm extraction was performed (see below). DNA was precipitated from the aqueous upper layer by adding 650 µl of isopropanol, leaving 15 minutes and then

spinning for 15 minutes, all at room temperature. After centrifugation the pellet was washed in 70% ethanol, dried and resuspended in distilled water.

Medium and large scale preparation of plasmid DNA

0.1 to 1 ml of plasmid bacterial culture was placed in 100 ml of LB containing 100 mg/ml ampicillin, and shaken at 37 °C overnight. The Haybaid Recovery midi or maxi-prep kit was then used to isolate the DNA.

DNA quantification and manipulation

DNA and RNA were quantified by spectrophotometry at 260 nm (optical density, OD=1 equates to 50 μg/ml double stranded DNA, 35 μg/ml single stranded DNA and 40 μg/ml RNA). The ratio between the readings at 260 nm and 280 nm provided an estimate of the purity of the nucleic acid (pure preparations of DNA and RNA should have OD_{260}/OD_{280} values of 1.8 and 2.0, respectively).

Phenol/Chlorophorm extraction

To remove proteins from nucleic acid solutions, a mixture of phenol:chlorophorm:isoamyl-alcohol (25:24:1 volume ration) was added in a 1:1 volume ratio to the DNA solution and vortexed for 1 minute. After a 3 minute centrifugation, the upper layer was transferred into a new microfuge tube and extracted with an equal volume of chloroform.

Precipitation

Concentration of DNA was performed by ethanol precipitation. 3 M NaOAc pH 5.5 (to a final concentration of 0.3 M) and 2.5 volumes of 100% ethanol were added to a DNA solution and left on dry ice for approximately 20 minutes. 1 μl of 10 mg/ml glycogen was

often used as a carrier if the DNA amount to be purified was too small to be visualised as a pellet at the bottom of the tube. Centrifugation at >20 000 g for 5-20 minutes was performed and the DNA pellet was then washed in 70% ethanol, dried and resuspended in TE or water.

Ligation and de-phosphorylation reactions

Intermolecular ligations were performed in small volumes, generally 20 µl for a total DNA content of 0.5 µg. Ligations were performed overnight at 14°C using T4 DNA polymerase (Gibco BRL) and the appropriate ligation buffer (Gibco BRL).

When the plasmid was capable of self-ligation, the compatible ends were dephosphorylated before use. Dephosphorylation of approximately 1 µg DNA was performed using alkaline phosphatase 0.1 u/µl (Boehringer) and alkaline phosphatase buffer (Boehringer) in 50 µl total volume. The reaction was incubated at 37°C for 20-30 minutes, followed by phenol/chlorophorm precipitation and further purification using the QIAquick Gel Extraction Kit protocol (without running the DNA on gel).

Agarose gel electrophoresis of DNA and RNA

DNA separation and size estimation were performed by agarose gel electrophoresis. Gels were prepared by dissolving agarose in 0.5 x TAE to a final concentration of 0.8% to 2% depending on the expected size of the DNA fragment. To visualise the DNA, 0.5 mg/ml ethidium bromide was added to the gel. DNA samples were mixed with 6X gel loading buffer and electrophoresis was performed at 5 to 20 V/cm of gel length, until the appropriate resolution was achieved. The resolved DNA was visualised using ultraviolet

light at 340 nm, and the size was estimated by comparison with known size markers such as the 1 kb size markers (Gibco BRL).

Purification of specific DNA fragments from gels

In order to purify DNA fragments of interest, DNA was subjected to agarose gel electrophoresis and the region of the gel containing the appropriate band was excised under ultra-violet light (365 nm). DNA was purified using the QAIquick Gel Extraction Kit protocol using a microcentrifuge, according to the instructions of the manufacturer (Qiagen).

Polymerase Chain Reaction (PCR)

PCR reactions were carried out using different versions of the *Thermus aquaticus* (Taq) DNA polymerase (AmpliTaq or KlenTaq, Perkin Elmer and Clontech, respectively), Vent (NewEnglandBiolabs) or Pfu (Stratagene) DNA polymerases according to the manufacturer's recommendations. Pfu DNA polymerase has a very low error rate and thus it was preferably used for applications requiring high-fidelity DNA synthesis, such as cloning. The reactions were set up using the buffers supplied in a total volume of 50 µl. dNTPs were added to a final concentration of 0.2 mM each, and primers were added to a final concentration of 0.4 µM. PCR reactions were performed on a Perkin-Elmer Thermal Cycler or Stratagene Robocycler 40.

DNA Sequencing

Generally, sequencing was performed with DNA obtained after medium or large-scale preparation of plasmid DNA.

(i) manual sequencing

Manual DNA sequence analysis was performed by the dideoxy chain termination method using a sequencing kit (Sequenase v.2, USB). 1 to 5 μg of DNA was denaturated for 5 minutes at room temperature in 0.2 mM EDTA/ 200 mM NaOH in 20 μl, followed by neutralisation with 2 μl of 2 M ammonium acetate pH 4.6 and precipitation with 60 μl of 100% ethanol. After centrifugation, the pellet was washed with 70% ethanol and resuspended in a suitable volume to be used in annealing and subsequent labelling and termination reactions of the sequencing procedure, which were performed according to the manufacturer's instructions. Sequencing reactions were loaded onto a sequencing gel (6% acrylamide/bis-acrylamide (19:1), 7 M urea, 0.8 or 1-fold TBE, 7 μl /ml of 10% ammonium persulfate and 0.35 μl/ml of TEMED) and run at 50 W, for approximately 2 hours. After electrophoresis, the gel was fixed in 10% methanol (or ethanol)/10% acetic acid for approximately 20 minutes. The fixed gel was dried on a vacuum drier and autoradiographed overnight at room temperature.

(ii) automated sequencing

Automated sequencing was performed on an ABI Prism 377 sequencing machine (Perkin Elmer Corporation) using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit. The samples were prepared according to the manufacturers instruction. For Data analysis ABI sequencing software was used

In vitro transcription

RNA was transcribed from constructs containing a promoter from bacteriophage SP6 or T7, which are highly specific promoters. The transcription method used allowed the synthesis of capped RNA, which is necessary for efficient translation. Transcription reactions were performed as described below (see also [Amaya, 1996 #52]:

10 µl	5 X Transcription buffer (Promega)
5 µl	0.1 M DTT
5 µl	10 mM rATP
5 µl	10 mM rCTP
5 µl	10 mM UTP
5 µl	1 mM rGTP
5 µl	5 mM Cap analogue (m7-G; New England Biolabs)
2.5 µl	RNase inhibitor (Promega)
5 µl	Linearised template DNA (1 µg/µl)
2.5µl	20u/µl SP6 (Boehringer) or T7 (Promega) RNApolymerase

The reaction mixture was incubated at 37°C for 30 minutes, after which 2.5 µl of 10mM rGTP was added and the reaction was incubated for a further 60 minutes at 37°C. 5 µl of RNAase-free DNAase I (Promega) was added to this reaction, to degrade the DNA template, and the reaction was further incubated at 37°C for 15 minutes. The mixture was then subjected to phenol/chloroform extraction followed by size exclusion chromatography using Nu-Clean R50 spin columns (Kodak) or Chroma Spin-30+DEPC-H_2O columns

(Clontech) to remove free nucleotides. Finally, the flow-through was ethanol precipitated, the pellet was resuspended in a suitable volume (generally 20 µl) and the RNA concentration was determined by spectro-photometry.

To determine the efficiency of translation of the synthesised RNA, synthetic RNA was usually translated using the rabbit reticulocyte system (Promega) and ^{35}S-methionine-labelled protein products were analysed by polyacrylamide gel electrophoresis. *In vitro* synthesised RNAs were used for injection into *Xenopus* embryos or as a template for synthesis of *in vitro* translated protein for electro mobility shift assays.

In vitro protein synthesis

Two approaches were used for *in vitro* translation: a programming system using RNA templates (Rabbit Reticulocyte Lysate System Nuclease Treated, Promega) or a programming system using DNA templates (T7/SP6 TNT Coupled Reticulocyte Lysate System, Promega). Small-scale ^{35}S-methionine labelled or large-scale unlabelled *in vitro* translations were performed for analytical or preparative purposes, respectively. Reaction conditions were as follows:

	Labelled	Unlabelled
RNA-based *in vitro* translation:	Small-scale	Large-scale
Denaturated RNA 1µg/µl or $_dH_2O$	0.2 µl	2 µl
RNase inhibitor 40 u/µl (Promega)	0.4 µl	1 µl
1 mM amino acids minus methionine (Promega)	0.4 µl	1 µl
Redivue L-^{35}S-methionine >1000Ci/mmol	1.6 µl	–

1 mM amino acids minus leucine (Promega)	_	1 µl
Reticulocyte Lysate, nuclease treated (Promega)	14 µl	35 µl
Distilled water	3.4 µl	10 µl

DNA-based *in vitro* translation

DNA 2 µl/µl or $_dH_2O$	0.5 µl	1 µl
RNase inhibitor (Promega)	0.4 µl	1 µl
1 mM amino acids minus methionine (Promega)	0.4 µl	1 µl
Redivue L-^{35}S-methionine >1000Ci/mmol	1.6 µl	_
1 mM amino acids minus leucine (Promega)	_	1 µl
TNT Reaction Buffer (Promega)	0.8 µl	2 µl
TNT T7 or SP6 RNA polymerase (Promega)	0.4 µl	1 µl
TNT Reticulocyte Lysate (Promega)	10 µl	25 µl
Distilled water	5.9 µl	18 µl

Western blot

Protein or tissue extracts were analysed in 10.0-17.5% acrylamide SDS-PAGE electrophoresis gels and subsequently blotted on a polyvinylidene difluoride membrane (Sequi-Blot™ PVDF protein sequencing membrane, Bio-Rad), previously permeabilised by submersion in methanol. The electrophoretic transfer was performed in 1X CAPS Buffer/10% methanol (10X CAPS Buffer stock contains: 22.1 g of 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) in 900 ml water, titrated to pH 11 with 2 N NaOH and filled up to 1 l with water) for 30 minutes at 200 mA constant current.

The PVDF membrane was pre-blocked with 5% milk powder (Marvel) in PBS for 1 hour at room temperature with moderate shaking. Then, it was incubated with 5 to 10 μg/ml of primary antibody in 0.5% milk powder/PBS overnight at 4°C. Anti-HA mouse monoclonal antibody (Boehringer Mannheim), anti-Myc mouse monoclonal antibody (9E10; Santa Cruz Biotechnology Inc.) or Anti-Xbra rabbit polyclonal antiserum (FB163) were used as primary antibodies. A series of 4 washes of 15 minutes each were performed using 0.1% Tween 20/PBS followed by a 1 to 2 hour incubation with the secondary antibody. Anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (Sigma) or Anti-rabbit IgG alkaline phosphatase conjugate (according to the primary antibody) were used as the secondary antibody in a 1:1000 dilution. The membrane was placed for approximately 2 minutes in Reaction Buffer (100 mM Tris pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Detection of alkaline phosphatase activity was assayed by overlaying the membrane with 3 or 4 ml of BM Purple Substrate (Boehringer Mannheim), which contains the substrates of the alkaline phosphatase reaction (Nitro Blue Tetrazolium Chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP)). The membrane was kept in the dark for 10 to 40 minutes until the staining developed. The reaction was terminated with Stop Buffer (100 mM Tris, 100 mM NaCl, 10 mM EDTA) and washed in distilled water to avoid formation of salt crystals during air drying.

Co-Immunoprecipitation

In preparation for the immunoprecipitation Protein A-Sepharose 6 MB beads (Pharmacia) were swollen in an equal volume of IP buffer (50mM Tris-HCl [pH7.5], 150mMNaCl, 1mM EDTA, 1% Triton X-100, 0.25% gelatin, 1mM PMSF, 0.02% sodium azide). 10μl of in vitro

translated extract was diluted with 90µl IP buffer and incubated with 10µg/ml αHA antibody for 1 hours at 4°C gently rotating. Then 10µl of the swollen Protein A-Sepharose beads were added and incubate gently rotating for another hour. The immune complexes were washed with the following sequence for 10 minutes each: IP buffer+0.2M NaCl ; IP buffer+0.40M NaCl, IP buffer+0.2M NaCl. For the washing steps the beads were collected on the bottom of the tube by pulse spinning, then the supernatant was carefully aspirated and new buffer applied. After the last washing step approximately 20µl of buffer was left in the tube and 5µl of 5x sample buffer for SDS page was added.

Electro mobility shift assays (EMSA)

Proteins used in EMSA were prepared by *in vitro* translation from synthetic capped RNA, as described above. Binding reactions contained 2 µl of *in vitro* translated protein, 1X binding buffer (75mM KCl, 0.25mM EDTA, 10mg/ml BSA, 1mM DTT, 0.1% NP-40, 1mM $MgCl_2$, 1mM PMFS. 10% glycerol, 25mM HEPES [pH 7.0]), 1-2 µg dIdC DNA (Sigma), 50,000-100,000 cpm probe in a total volume of 12 µl. Complexes were allowed to form at room temperature for 10-15 min before adding probe and were incubated for 15-20 min after addition of probe. For antibody shift analyses, 1 µg of monoclonal anti-HA antibody (Boehringer), was added to the reactions and incubation was continued for an additional 10-15 min. Complexes were resolved on a 4% polyacrylamide gel. Gels were run initially at 300 mV for 10 minutes and then at 100-200 mV for 2-3 hours at 4°C. The palindromic T-probe used for Fig 4.8 was annealed for 10 minutes at 90°C and cooled slowly to room temperature, after which it was labelled by 3' filling with ^{32}P-dATP (3,000 Ci/mmol) and dCTP (3,000 Ci/mmol) using the Klenow fragment (Promega).

The sequence of the oligo nucleotides constituting the palindromic T-probe were as follows: **5'**: ATTAGTCACACCTAGGTGTGAAGAGCC

3': GGCTCTTCACACCTAGGTGTGA

In vitro MAP kinase assays

Affinity purified protein at a final concentration of 2.5µM was incubated with 1x MAPK buffer (New England Biolabs: 50mM Tris-HCl [pH7.5@25°C]10mM $MgCl_2$, 1mM DTT, and 1mM EGTA) and supplemented with 100µM ATP and 250µCi γ^{32}P-ATP (NEN life sciences). All reactions for different time points were performed in the same tube in 20µl per reaction. MAP kinase (Erk2, New England Biolabs) was added at time point 0 at one unit per reaction. At the final time point for an individual reaction 10 µl of the reaction mix were transferred into a prepared reaction tube containing 20µl of 500mM DDT and 1.5% SDS and frozen immediately.

For analysis of γ^{32}P incorporation, the samples were loaded on a 16% acrylamid gel and separated at 300V. To ensure equal loading the gel was stained with Coomassie blue in acidic acid/methanol. After staining the gel was dried and exposed to X-ray film between 10 minutes and over night.

Site-directed Mutagenesis

The introduction of specific mutations into plasmids was performed using the QuickChange™ Site directed mutagenesis kit from Stratagene® This method makes use of

the high fidelity and nonstrand-displacing action of the *Pfu* DNA polymerase. A complementary pair of 5' and 3' oligonucleotides carrying the mutation or internal deletion serve as primer for the *Pfu* DNA polymerase to amplify the whole plasmid in a temperature cycling reaction incorporating the mutated oligonucleotide. The reaction results in nicked strands, thus only the parent plasmid is amplified, which avoids the amplification of random mutations. After the cycling reaction the DNA is treated with the enzyme *Dpn*I, which is specific for methylated and semi-methylated DNA, and so digests the parent strands, but not the newly synthesised DNA (DNA isolated from almost all E. coli stains is dam methylated, and thus susceptible to *Dpn*I digestion). The resulting DNA was directly transformed into the supercompetent Epicurian Coli® XL1-Blue. Preparation of DNA from three or four colonies was usually sufficient to obtain a plasmid carrying the desired mutation. All mutations were confirmed by automated sequencing. Initially the whole coding-region or long stretches of the promoter regions were sequenced to confirm the integrity of the region, however, in no case a random, unspecific mutation was detected. Thus, in later experiments only a single sequencing reaction spanning 600-800 nucleotides around the mutated site was used to confirm the mutation.

2.2. Plasmid constructs

2.2.1 Xbra promoter constructs

All *Xbra* promoter constructs used in this study, with exception of *Xbra4.1-GFP*, are based on the plasmid pXbra-2.1-GFP (pXbra2-GFP, Latinkic *et al.*, 1997), which contains the *Xbra* promoter sequence between nucleotide –2165 and +48 of *Xbra2* upstream of a GFP

reporter gene. The *Xbra2* gene is a pseudo-allele of the originally cloned *Xbra* and has an identical expression pattern and identical induction properties with *Xbra* (Latinkic *et al.*, 1997). The promoter of the original *Xbra* gene has not been isolated yet. I refer to the promoter of the *Xbra2* gene as *Xbra* promoter in this study. The *Xbra-2.1-GFP* construct has been created by replacing the Luciferase reporter gene of the pGL3-basic plasmid (Promega) with a GFP reporter gene (Zernicka-Goetz *et al.*, 1996, Latinkic *et al.*, 1997). The Xbra-4.1-GFP construct contains the 2165 bp 5'-flanking Xbra region, but also includes the first Xbra exon, the first intron and part of the second exon fused in frame with the GFP coding region. This construct was kindly provided by Branko Latinkic.

Xbra promoter deletion constructs

Xbra-970-GFP:

This construct has been created by excising a PstI/ NdeI fragment from the *Xbra-2.1-GFP* plasmid and re-ligating the plasmid after filling in the ends with Klenow DNA polymerase. *Pst*I cuts internal of the Xbra sequence, while the *Nde*I site is part of the multiple cloning site (MCS) upstream of the promoter region. Thus *Xbra-970-GFP* contains the *Xbra* promoter sequence from nucleotide –970 to +48. The 48 nucleotides of *Xbra* untranslated 5' sequence, downstream of the transcription start site has been kept intact in all the deletion constructs.

Xbra-381-GFP, Xbra-300-GFP, Xbra-231-GFP, Xbra-150-GFP:

All these constructs were created by replacing the 2.1 kb *Xbra* 5' flanking region in *Xbra-2.1-GFP* with the shorter *Xbra* 5' flanking region of the deletion construct at the *Mlu*I/*Xho*I

restriction sites in the MCSs outside the promoter sequence. The promoter regions for the deletion constructs were amplified using *Xbra-2.1-GFP* as a template.

The 3' primer was identical for all the constructs and contained a *Xho*I restriction site:

Xho-Xbra48: CCGCTCGAGCAGGTAGTAAATCC

The 5' primers were specific for the deletions and contained a *Mlu*I site (underlined):

MluI-Xbra-381: CGAACGCGTCATCTGCCATTATACCA

MluI-Xbra-300: GGAACGCGTCAGTTCTTACTGGATG

Mlu-Xbra-231: CGAACGCGTCTATATCAAAGAGCTG

Mlu-Xbra-150: GGAACGCGTCATAGAGCTCTCTGG

Promoter constructs containing point mutations

For the point mutations I have used the Stratagene® QuikChange™ Site-Directed Mutagenesis Kit, which uses a pair of complimentary oligonucleotides containing the desired point mutations.

I created point mutations in two promoter constructs described in this study:

Xbra-2.1AB^{mut}-GFP:

5': CCTCTGACTTGCAATT**G**AATTCCCAGGAT**GC**TCATAGAGCTCTCTG

3': CAGAGAGCTCTATGA**GC**ATCCTGGGAATT**C**AATTGCAAGTCAGAGG

This mutation disrupts two homeo domain binding sites at the positions –154, -155 and –169 in the context of the 2.1 kb promoter.

Xbra-2.1A^{mut}-GFP:

5': CCTCTGACTTGCAATTGAATTCCCAGGATTATCATAGAGC

3': GCTCTATGATAATCCTGGGAATTCAATTGCAAGTCAGAGG

disrupts and antennapedia type homeodomain binding site in position –169 in the context of the 2.1 kb promoter.

The following promoter constructs with point mutations existed already in the background of the pGL2,Luc vector and needed only to be subcloned into the background of the pGL3.GFP vector. This was done by excising the fragment at the *Hind*III/*Mlu*I restriction site and cloning it at the same site into the pGL3.GFP vector.

Xbra-2.1TATA/SRFmut-GFP: from *Xbra2.1M2-Luc*, (Branko Latinkic)

Xbra-381AB^{mut}-GFP: from Xbra381AB-Luc (Latinkic *et al.*, 1997)

Xbra-381B^{mut}-GFP: from Xbra381B-Luc (Branko Latinkic)

Xbra-2.1mut1-GFP: from *Xbra-2.1mut1-Luc* (Remacle et al., 1999)

Xbra-2.1mut2-GFP: from *Xbra-2.1mut2-Luc* (Remacle et al., 1999)

Xbra-2.1mut3-GFP: from Xbra-2.1mut2-Luc (Remacle et al., 1999)

Xbra-2.1mut4-GFP: from *Xbra-2.1mut2-Luc* (Remacle et al., 1999)

The following constructs with point mutations had been done already for the background of the 2.1kb promoter and were amplified from these with the primers for the *Xbra-381-GFP* deletion constructs:

Xbra-381TATA/SRFmut-GFP

Xbra-381A^{mut}-GFP

2.2.1 Xbra protein constructs

Xbra N-terminal deletion constructs

N-terminal deletion constructs were based on the pSP64TBX-XbraHA (Masa Tada, Division of Developmental Biology, NIMR, London). pSP64T is a plasmid optimised for in vitro transcription using SP6 polymerase (Promega). Masa Tada modified it to contain additional MCSs and two HA-tags followed by stop codons and has cloned the coding region of *Xbra* in frame upstream of the HA tags. For my purpose I replaced the full coding region of *Xbra* with shorter fragments encoding N-terminal truncations of the protein. The following primers were used to amplify the fragments.

Xbra3'EV: GTTGATATCGGACTGATGGTGGCG

The 3' primer was the same for all N-terminal deletion constructs. It was directed to the 3' end of the coding region, but contained an *Eco*RV site (underlined) instead of the stop codons to clone it in frame with the HA tags

Bgl-Xbra-75: GTTAGATCTGCCATGCTCCAGGCTGGCAG

Bgl-Xbra-150: GTTAGATCTGCCATGGACGAGGTTCAAGG

Bgl-Xbra-177: GTTAGATCTGC*CATGCA*GAGAATGATCACTAG

Bgl-Xbra-200: GTTAGATCTGCCATGACGAATGTTTCCAG

Bgl-Xbra-225: GTTAGATCTGCCATGAGCATGTCGGGCCTG

The 5' primers contain a *Bgl*II site (underlined) to clone it into pSp64T and a GCC sequence upstream of the ATG to enhance translation efficiency.

C-terminal Xbra deletion constructs

C-terminal deletions were designed with a myc-tag at their C-terminal end. Frank Conlon kindly provided me with a pBSK-myc plasmid. I made use of this plasmid as an intermediate to first clone Xbra deletion constructs into a plant-ended *Cla*I site and a *Hind*III site in frame to the myc-tag. Then the myc-tagged *Xbra* fragments were released via a *Spe*I site from the pBSK and via a *Bgl*II site that has been added in the design of the 5' primer. These are the optimal restriction ends to clone it into pSP64T.

The 5' primer was:

Bgl-Xbra5': GTTAGATCTGCCATGAGTGCGACCGAGC

The *Bgl*II site is underlined

The 3' primer were:

Hind-Xbra226: GTTAAGCTTGTCTTTATAATCATTTC

Hind-Xbra250: GTTAAGCTTCCTGGATCCCAATG

Hind-Xbra280: GTTAAGCTTTAGAGATGAGTATCG

Hind-Xbra303: GTTAAGCTTGACAACCACCGCTGGAA

HindXbra387: GTTAAGCTTATGTGAAAGAGACGAG

The *Hind*III site is underlined.

Construct for expression in bacteria

pET-22BXbraΔ303

XbraΔ303 was cloned into the *Nae*I/*Xho*I sites of pET-22B (Novagen) resulting in a 6xHIS tag fused to the c-terminus of the protein for affinity purification. This cloning step was performed by Richard Tyrell (Division of Protein Structure)

Xbra protein constructs with point mutations

Point mutations were induced in each of the five putative MAP kinase sites. The mutations were introduced into the pSP64TXbraHA construct and the pET-*22BXbraΔ303* construct for in vitro transcription and bacterial expression respectively. The primers were as follows:

MK1-5': GTTTATATCCACCCAGACGCACCCAACTTTGG

MK1-3': CCAAAGTTGGGTGCGTCTGGGTGGATATAAAC

resulting in **S127 → A127.**

MK2-5': CAGCTCCTTACCCCGCTCCGTACACTCACAGAAAC

MK2-3': GTTTCTGTGAGTGTACGGAGCGGGGTAAGGAGCTG

resulting in **S290 → A290.**

MK3-5': CACTCACAGAAACAATGCTCCAAACAATTTAGCAG

MK3-3': CTGCTAAATTGTTTGGAGCATTGTTTCTGTGAGTG

resulting in **S298 → A298.**

MK4-5': GGTGGGTCATTATGTGCGCCCAATCCACAC

MK4-3': GTGTGGATTGGGCGCACATAATGACCCACC

resulting in **S255 → A255**

MK5-5': CTCTCTCTATCGGCACCTCATGGCTGTGAGCG

MK5-3': CGCTCACAGCCATGAGGTGCCGATAGAGAGAG

resulting in **S270 → P270**

2.3. Embryos and Embryo Manipulations

Xenopus transgenesis

The protocol for *Xenopus* transgenesis (Amaya and Kroll, 1996) involves the following steps; (1) Sperm nuclei are incubated with linearised plasmid DNA. (2) After a short incubation, a high-speed interphase egg extract and a small amount of the restriction enzyme used for plasmid linearisation are added to the sperm nuclei and plasmid mixture. The extract partially de-condenses the sperm chromatin, allowing the restriction enzyme to lightly cleave the sperm DNA. Integration of the plasmid can occur, but the extract does not promote replication. (3) After the plasmid-treated nuclei are incubated for a brief period in the interphase extract, the mixture is diluted 50-100 fold to ensure that approximately one nucleus is transplanted into an unfertilised egg in a 5 nl volume. This results in a normal diploid embryo containing the integrated transgene with the potential to be expressed in all cells of the embryo.

Sperm nuclear preparation

A male *Xenopus laevis* was anaesthetised in a litre of 0.1% Tricaine (MS222, aminobenzoic acid ethyl ester) for 20 minutes and then decapitated and pithed. The testes were removed, washed three times with ice cold Marks Modified Ringers (MMR: 100mM NaCl, 2mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 5mM HEPES pH 7.5), and two times in ice cold Nuclear Preparation Buffer (NPB: 250mM sucrose, 15mM HEPES, 1mM EDTA, 0.5mM Spermidine trihydrochloride, 0.5mM Spermine tetrahydrochoride, 0.1mM Dithiothreitol) containing protease inhibitors (10μg/ml leupeptin and 0.3mM phenylmethylsulphonyl fluoride

(Boehringer)). The cleaned testes were then macerated with watchmakers forceps, resuspended in 10ml of ice cold NPB, and forced through four layers of cheesecloth into a 15ml Falcon tube. To wash the sperm, they were centrifuged at 3000rpm for 10 minutes at 4°C, and then resuspended in fresh NPB (ice cold). The sperm were repelleted by a further 3000rpm spin for 10 minutes at 4°C, then resuspended in 1ml of NPB at room temperature, mixed with 50µl 10mg/ml lysolecithin (Sigma), and incubated on the bench for 5 minutes. The lysolecithin removes the outer membrane from the sperm but leaves the nucleus intact. After 5 minutes, 9mls of ice cold NPB supplemented with 3% BSA and protease inhibitors was added and the sperm nuclear suspension was spun down (3000rpm for 10 minutes at 4°C). The nuclei were then resuspended in 5ml of ice cold NPB containing 0.3% BSA (no protease inhibitors), and repelleted as before. Finally, the sperm nuclei were resuspended in 500µl of Sperm Storage Buffer (SSB: NPB, 0.3% BSA containing 30% glycerol). The concentration of the sperm stock in SSB was determined by Hoechst staining and counted in a haemocytometer. From one male, I typically obtained 500µl of $1X10^8$ sperm/ml stock. Sperm nuclei were stored in SSB at 4°C and used for transplantations for up to 48 hours.

Interphase egg extract preparation

The night before extract preparation, twelve female *Xenopus laevis* were placed into separate buckets containing 1xMMR and injected with 500U of Chorulon (Sigma) to induce egg production. Next morning the eggs laid by each frog were screened for quality and dejellied in 2% L-cysteine HCl in XB salts (100mM KCl, 0.1mM $CaCl_2$, 1mM $MgCl_2$). The eggs were washed four times in XB salts containing 10mM HEPES and 50mM sucrose, at which time any lysing or activated eggs were removed. Eggs were then washed twice in CSF XB (XB salts modified to include 10mM potassium HEPES pH 7.7, 50mM Sucrose,

5mM EGTA pH7.7, final $MgCl_2$ concentration of 2mM). Protease inhibitors (10mg/ml leupeptin and pepstatin), were also added to the CSF XB. Using a wide bore pipette, eggs were transferred to 14 X 95mm ultraclear centrifuge tubes (Beckman). As much CSF XB as possible was removed from the top of the tubes and replaced with 1ml of Versilube F-50. The tubes were then spun for 60 seconds at 1000rpm and 30 seconds at 2000rpm. This spin displaces the remaining CSF XB surrounding the eggs, which was replaced with Versilube. The tubes were then spun at 10000rpm for 10 minutes (2°C) in a swinging bucket rotor to crush the eggs and separate them into three layers; lipid (top), cytoplasm (middle), and yolk (bottom). The cytoplasmic layer was collected with an 18 gauge needle and syringe. Protease inhibitors were added and the cytoplasm was recentrifuged (10 min, 10000rpm at 2°C) to remove any traces of yolk. $CaCl_2$ was added to a final concentration of 0.4mM and the extract was incubated at room temperature for 15 minutes. The $CaCl_2$ releases the CSF XB-mediated cell cycle arrest and allows the extract to progress into interphase. Next, the extract was centrifuged at 70000rpm for 90 minutes at 4°C using a TL-100.3 rotor and a Beckman tabletop TL-100 ultracentrifuge. The cytosolic layer was collected through the top of the tube, transferred to fresh TL-100 tubes and spun for an additional 20 minutes. Aliquots of 25µl were frozen in liquid nitrogen and stored at -80°C until use. From 12 female frogs I typically achieved 1-2mls of high speed interphase extract.

Transplantation needles

Needles used for nuclear transplantations were made from 30µl borosillate micropipettes (Drummond) and pulled on a Campden Instruments needle puller (model 763). The micropipettes were pulled in low heat to produce a gently sloping needle tip. Needles were clipped with watchmakers forceps to produce a beveled tip of 60-75µm diameter using the ocular micrometer of a dissecting microscope for measurement. Prior to use in

transplantations, needles were coated with Sigmacote (Sigma SL-2), then rinsed with distilled water.

Nuclear transplantations

To obtain a supply of unfertilised eggs four female *Xenopus laevis* were injected with 500U of Chorulon and incubated at 18°C for 12-16 hours before transplantations. For the reaction, 4µl of sperm stock in SSB was mixed with 5µl of linearised reporter construct and incubated at room temperature for 5 minutes. 1U of NotI in 0.5µl was added along with 2µl of 100mM $MgCl_2$ to aid NotI enzyme action. To swell the sperm, and allow transgene integration, 25µl of high speed interphase extract was added, and the reaction mixed by pipetting. While the sperm were swelling, about 1000 eggs were collected and dejellied in 2.5% L-cysteine-HCl (pH 8 in MMR). Eggs were then transferred into agarose-coated injection dishes containing 0.4 X MMR containing 6% ficoll. After incubating the sperm with egg extract for 10-15 minutes, they were diluted with 200µl Sperm Dilution Buffer (SDB: 250mM sucrose, 75mM KCl, 0.5mM Spermidine trihydrochloride, 0.5mM Spermine tetrahydrochloride, 0.1N NaOH per 20mls to pH 7.3-7.5), yielding a final concentration of approximately 2 sperm nuclei / 5nl. Using a piece of Tygon tubing attached to a 200µl tip, the sperm solution was gently mixed by pipetting and then backloaded into a needle. The needle was attached to the injection apparatus and the eggs were each injected with a 5nl volume.

Transplantation apparatus

For transplanting the sperm suspension into the unfertilised eggs I made use of a Drummond Nanoject injector (3-00-203X7), which can deliver an exact amount of 5nl by a positive displacement mechanism. This has proven a very efficient and simple way of transplantation because up to 1000 eggs could be injected within 15 minutes. This was of advantage, because experience showed that after 15 minutes of transplantation the rate of normal development following transplantation decreased drastically.

Transgenic embryo selections

When cleaving transplantation embryos reached the 4-cell stage they were separated from uncleaved eggs and moved to a separate dish containing 0.1 X MMR containing 6% ficoll and 50μg/ml gentomycin. Normally cleaving embryos represent those which have been injected with a single nucleus from the diluted sperm solution, whilst uncleaved embryos have only received buffer. Eggs injected with more than one sperm nucleus divide at the first time of cleavage abnormally into three or more cells. These polyspermic eggs were discarded along with the uncleaved eggs at this stage. On average 5-25% of embryos showed a normal cleavage pattern. The selected embryos were then stored overnight in batches of 15-20 embryos in medium tissue culture dishes at 15°C. Typically, approximately 30-60% of embryos that developed normally during cleavage stages also completed gastrulation, and further 20-30% of embryos developed to tadpole stages if allowed.

Linearisation of DNA for transgensis

In a previous study (Kroll and Amaya, 1996) it was suggested to linearise DNA with the same enzyme that is then used in the incubation of nuclei with interphase extract. However, while in this study the protocol was standardised for the use of *not*I during the incubation of the nuclei, there was no significant influence on the efficiency of transgene expression depending on the enzyme used for linearisation. In this study *Mlu*I was mostly used to linearise the plasmids. Alternatively, mostly for the shorter fragments, the vector sequence was removed by releasing the promoter and reporter at the sites *Mlu*I/*Sal*I.

Analysis of transgenic embryos

The expression pattern of any individual promoter construct in transgenic embryos was analysed by in situ hybridisations against the reporter gene (GFP in all cases presented in this thesis). Transgenic frequency, as judged by expression, varied between 40% and 90%. A minimum of 25 expressing embryos were analysed per construct at a presented stage unless stated differently in the text. Percentages were always calculated against expressing embryos.

Obtaining Xenopus embryos by artificial fertilisation

In addition to transgenesis, *Xenopus laevis* embryos were also obtained by artificial fertilisation as described by Smith and Slack (1983) Briefly, *Xenopus* embryos were obtained from adult females that had been injected 12 hours previously with 500-1000 units of human chorionic gonadotrophin, and transferred to a 90 mm Petri dish. The eggs were fertilised by rubbing them with testes dissected from a sacrificed male. Males were sacrificed by decapitation and disruption of the spinal cord and the testes were dissected

and submersed in L15 media (Sigma) at 4°C for 2 or 3 days. Five minutes after fertilisation the eggs were flooded with 10% Normal Amphibian Medium (NAM; Slack, 1984. The embryos were de-jellied using 2% cysteine hydrochloride (pH 7.9-8.1), and staged according to Nieuwkoop and Faber Nieuwkoop and Faber, 1975.

Microinjection of Xenopus embryos

For injection with RNA, fertilised embryos were transferred to 0.3 x MMR containing 4% Ficoll into agarose dishes lined with 1% agarose. All injections were performed with the Drummond Nanoject injector (3-00-203X7), which delivers accurate volumes in 5 nl increments. For the experiments confirming protein stability 500 pg of RNA encoding the individual constructs was injected at the two cell stage into each of the cells. For the cellular localisation study 500 pg of RNA was injected into one cell of the 8 cell embryo.

Preparation of embryo extract

To test the stability of the deletion constructs, injected embryos were harvested at stage 9.0. Only the animal hemisphere was taken to prepare the extract, because the high protein and lipid content of the vegetal hemisphere can interfere with immuno-detection. The vitelline membrane surrounding the embryo was removed manually using sharpened number 5 watchmakers forceps (supplied by BDH). A square of tissue from the animal-most 20-25% region was cut by using the forceps as scissors. 10 of these 'animal caps' were collected and lysed in 50 µl homogenisation buffer (150 mM Tris-[pH8], 25% glycerol, 50 mM KCl, 2 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 25 µg/ml aprotenin, 25 mM ß-glycerolphosphate, 2 mM Sodium vanadate), The lysate was cleared by centrifugation for 10 minutes at 10.000 rpm. The supenatant was transferred into a new tube and stored at –80°C.

Whole mount antibody staining

For whole mount antibody staining to detect cellular localisation of deletion constructs, blastula stage embryos were transferred into 75% NAM were the viteline membrane was removed. The embryos were fixed in MEMFA for 1 – 2 hours gently shaking at 4° C. After fixation they were washed in 100% Methanol for 2x 10 minutes, after which they were either stored at-20°C or further processed. To bleach the pigmented animal pole region, the embryos were transferred to 70% Methanol/10% H_2O_2 and kept overnight on a light box. The next morning they were rehydrated in subsequent washes of 70% methanol/$_dH_2O$, 50% methanol/ $_dH_2O$, 30% methanol/PBS for 5 minutes each, and rinsed for further 2x10 minutes in PBS.

Following rehydration they were incubated in Glycin (pH7-8) for 30 minutes, again washed in PBS for 30 minutes, transferred to PBSMT (PBS+0.5% milk powder+0.1% Triton X100) and washed in this solution 4x 10 minutes. In order to block unspecific epitopes the milk powder concentration was increased to 5% for another hour. The primary antibody was added in to PBSMT+5%milk powder and incubated over night. The following day the samples were washed 5x 1 hour in PBSMT and again blocked for 1 hour in PBSMT +5% milk powder. The secondary antibody (anti mouse or anti rabit) was added with the same solution and incubated overnight at 4°C. Like after the primary antibody the samples are washed 5x for 1 hour in PBSMT. For detection PBSMT was replaced with 1ml PBS/DAB (10mg DAB tablet was dissolved in 30ml dH_2O, then 3.3ml 10x PBS, 330µl 10% Triton and 10µg NiCl was added). After 10 minutes rinse the solution was replaced with fresh 1 ml PBS/DAB and 1µl 30% H_2O_2 was added and mixed as hard as possible, without damaging the embryos. When the staining had reached a satisfactory level, the embryos were fixed in MEMFA for 30 minutes.

In case the embryos had been co-injected with fluorescine dextran lysate another wash of 4x 15 minutes PBSMT and 1 hour block in PBSMT + 5% milk powder followed. The embryos were incubated in anti-fluorescine-AP Fab fragments (1:200) (Boerhinger Mannheim) over night at 4°C. Washes were like the days before in PBSMT 5x 1 hour. After the washes the embryos were rinsed in ice cold 100mM Tris/0.1% Tween at 4°C. During this time 1 fast red tablet (Boehringer Mannheim) per 2 ml Tris/0.1% Tween was dissolved (vortexed at room temperature, then on ice). Staining was done at 4°C. When staining was intense enough without covering the Xbra signal, the embryos were again fixed in MEMFA for 30 minutes.

To visualise internal cellular localisation, embryos were cleared in rising concentrations of Glycerol (30%, 50%, 70%, 80%, 90%) for at least 10 minutes each. After clearing a stained piece of the embryo was cut out, washed in 90% glycerol and transferred onto another drop o a glass slide. A cover slip was put on top and the tissue was carefully flattened.

Whole mount in situ hybridization

Whole mount *in situ* hybridization was basically performed as described in Jones and Smith, 1999. Gastrula stage embryos were removed from their vitelline membranes, a hole was made in the blastocoel to improve penetration of solutions, and they were fixed for 2 hours at 4°C in MEMFA (fresh formaldehyde at 3.7% in 1X MEM salts) before being transferred to methanol for long-term storage at −20°C. All procedures were performed in

5-ml screw top glass vials (Phase Separation). In all solutions containing water, water filtered to 0.2 micron (SPH_2O, Romil Ltd.) was used.

Embryos were rehydrated by taking them through a methanol/water (and PBS) series starting with 75% methanol in water, 50% methanol in water and then 25% methanol in PBS for about 5 minutes each. Embryos were then washed 3 times in PBS/Tween 0.1%. Proteinase K (Boehringer) at 5 µg/ml is then added, to increase penetration by degradation of surface proteins, and embryos were left for 10 to 20 minutes at room temperature. The timing of proteinase treatment was monitored carefully, because long exposures can damage the embryos. Embryos were again washed in PBS/Tween 0.1% and then treated with 4 ml of 0.1M triethanolamine pH 7.7, 5 minutes twice, to acetylate proteins (0.1 M triethanolamine solution was prepared previously by diluting the suitable amount of triethanolamine, adjusting the pH with HCl and then filtering with a Sartolab V500 filter). Without removing the triethanolamine solution, 10 µl of acetic anhydride was added and incubated for 5 minutes twice. This treatment blocks the activity of endogenous phosphatases. Again, the embryos were washed with PBS/Tween 0.1% for 5 minutes twice. To fix, embryos were post fixed in MEMFA for 20 minutes. The embryos were washed 5 times with PBS/Tween 0.1%, pre-hybridised with 1 ml of Hybridisation solution for 4 hours at 60°C and, finally, they were hybridised overnight at 60°C in 500 µl of this solution with 2 µl of denaturated dioxigenin (DIG)-labelled probe. The Hybridisation solution was: 50% deionised formamide, 5X SSC pH 6, 200 µg/ml t-RNA, 100µg/ml heparin, 1X Denhard'ts (50X stock contains 1 g polyvinylpyrrolidone, 1 g Ficoll and 1 g BSA made up to 100 ml with SPH_2O), 0.1% Tween 20, 0.1% CHAPS and 5 mM EDTA.

On the next day, the embryos were washed using a series of washing solutions with decreasing amounts of formamide: 50% formamide/5X SSC/0.1% CHAPS (10 minutes at

60°C), 25% formamide/2X SSC/0.1% CHAPS (10 minutes at 60°C), 2X SSC/0.1% CHAPS (30 minutes at 60°C twice), 0.2X SSC/0.1% CHAPS (30 minutes at 60°C twice). After the washes, embryos were treated with maleic acid buffer (150 mM NaCl, 0.1 M maleic acid pH 7.5) in 0.1% triton (MABT) for 5 minutes at room temperature twice. The MABT solution has a suitable pH for incubation with anti-DIG antibody and should be prepared with a non-ionic detergent such as Triton to avoid nonspecific interference with the antibody binding reaction. Before adding antibody, the embryos were treated with freshly prepared 2% blocking reagent (Boehringer)/10% lamb serum (heat inactivated)/MABT, for 1 hour at room temperature. Finally, embryos were incubated overnight at 4°C with gentle rotation with a 1:4000 dilution of anti-dioxigenin (DIG) Fab fragments conjugated with alkaline phosphatase 150u/200µl (Boehringer Mannheim). On the following day, embryos were washed 4 times for 1 hour at room temperature in MABT. Then, they were treated with alkaline phosphatase buffer (0.1 M Tris.HCl pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$, 0.1% Tween 20 and 2 mM levamisol freshly added) followed by 1 ml of BM purple alkaline phosphatase substrate (Boehringer Mannheim). When the staining was satisfactory the embryos were fixed in MEMFA for 30 minutes at room temperature and transferred to 100% Methanol. In case clearing was necessary, the embryos received two additional 10 minute washes in 100% Methanol and were then transferred to a 2:1 solution of Benzyl Alcohol: Benzyl Benzoate (Sigma).

The following probes were used for in situ hybridisation:

***DIG-labelled antisense GFP*:**

in vitro transcribed in presence of DIG nucleotides (Boehringer Mannheim) with T3 polymerase from a pBSK-GFP plasmid (kindly provided by Elena Casey) linearised with *Hind*III.

DIG-labelled antisense Xbra:

in vitro transcribed in presence of DIG nucleotides (Boehringer Mannheim) with T7 polymerase from the plasmid pXT1 (Smith *et al.*, 1991) linearised with *Stu*I.

Antibody staining of sections

All manipulation before embedding in wax were carried out in 5 ml screw top glass vials (Phase Separation). Embryos were fixed with MEMFA over night at 4°C, followed by two washes in PBS. In preparation for sectioning they were de-hydrated in a series of Ethanol washes (35%, 50%, 80%, 10% for 5 minutes each), followed by two 10 minutes washes in Histoclear (national diagnostics) and 3 x 20 minutes washes in liquid paraffin at 50° C. The embryos were then individually embedded in paraffin and stored at 4°C. 10 µm sections were cut on a microtome (Reichert-Jung).The sections were mounted onto a glass slide in a 50°C water bath and dried over night in 50°C oven.

The following washes were performed in glass boxes: The slides were 5 minutes incubated in Histoclear in order to remove the paraffin, then dehydrated in a series Ethanol washes (100%, 95%, 85%, 70%, 50%, 35% for 1 minute each; PBS + 0.1% Tween (PBST) for 10 minutes). Then the slides were transferred to a 'wet chamber' (a plastic box were the rested on plastic moulds above a water filled surface). The sections were blocked with 10% goat serum in PBST for 30 minutes and then incubated in primary antibody for 1

hour in the same solution. The unbound antibody was washed off in 3x 10 minutes washes in glass boxes, before the slides were transferred back to the wet chamber and blocked again with PBST + 10% goat serum. HRP-conjugated secondary antibody was applied for another hour in PBST +5 % goat serum. Three 10 minutes washes in PBST followed in glass boxes. For detection of the bound antibody, the sections were first incubated in PBST/DAB for 10 minutes and then with PBS/DAB +0.03% H_2O_2 until staining appeared. The glass slides were rinsed in PBS and mounted in Gurr Aquamount (BDH).

3. REGULATION OF THE *XBRA* PROMOTER

3.1 Introduction

The regulation of *Xbra* is of particular interest for the understanding of mesoderm formation for two reasons: (i) Its activation is a direct response to mesoderm inducing factors (Smith *et al.*, 1991). (ii) It is one of the first genes during embryonic development whose expression is exclusively confined to the prospective mesoderm (see Chapter 1; Wilkinson *et al.*, 1990, Smith *et al.*, 1991).

It seems the (ii) should follow from (i). However, I will discuss below why the combination of both points is a relatively unique feature for *Xbra* that may mark its special role in mesoderm formation. *Xbra* can be ectopically activated by several different signalling pathways implicated in mesoderm induction. It is ectopically activated in the animal hemisphere by molecules belonging to the TGFß and FGF families, such as BMP4 (Stennard *et al.*, 1996), activin (Smith *et al.*, 1991), Vg1 (Dale *et al.*, 1993; Thomsen and Melton, 1993), nodal (Jones *et al.*, 1995), Derriere (Sun *et al.*, 1999) and eFGF (Isaacs *et al.*, 1994). It is also activated by components of their signalling pathways, such as Smad1 (Armes and Smith, 1997), Smad2 (Baker and Harland, 1996) and the MAP kinase pathway (LaBonne *et al.*, 1995; Gotoh *et al.*, 1995; Umbhauer *et al.*, 1995). For activin and FGF it has been shown that this activation is direct in the sense that it can occur in the presence of the protein synthesis inhibitor cycloheximide (Smith *et al.*, 1991). Addition of FGF but not activin leads in addition to ectopic activation of *Xbra* in the vegetal hemisphere (Cornell *et*

al., 1995). Somehow, all these signalling pathways have to be integrated at the *Xbra* promoter to result in the proper expression of *Xbra* in the marginal zone.

Matters are made more complex by the fact that several of the signalling pathways that can activate ectopic *Xbra* expression are actually active in these places. BMP is present at high levels in the animal hemisphere during gastrulation (Fainsod *et al.*, 1994), as is MAP kinase signalling in the vegetal hemisphere (LaBonne and Whitman, 1997). In addition, other genes that are activated by mesoderm inducing factors, such as *Xwnt-8* (Christian *et al.*, 1991c; Smith and Harland, 1991), *eomes* (Ryan *et al.*, 1996), *Mix.1* (Rosa, 1989), *chordin* (Sasai *et al.*, 1994), *gsc* (Cho *et al.*, 1991) and *Xvent1* (Gawantka *et al.*, 1995) are not confined to the prospective mesoderm but also include domains of expression in prospective endoderm or ectoderm (Lemaire and Gurdon, 1994; Zorn *et al.*, 1999). How then are the sharp boarders created that confine the presence of Xbra to the newly formed mesoderm?

Known regulatory sequences in the *Xbra* promoter

To find out more about the regulation of *Xbra* I decided to search for sequences in the *Xbra* promoter that are responsible for its spatial and temporal expression pattern. Previous studies of the promoter have defined a region containing an activin and FGF response element to 150 bp (-231 to –381) in the 5' flanking *Xbra* sequence (Latinkic *et al.*, 1997). This study showed also that a combination of homeodomain binding sites in the proximal promoter region (-153 to –175) can confer the repression of Xbra at high levels of activin, which are thought to be present in the vegetal region of the embryo. The homeodomain binding sites have been shown to bind Gsc, Otx-2 and Mix.1 protein *in*

vitro, all of which can suppress endogenous *Xbra* expression when overexpressed in the embryo (Latinkic *et al.*, 1997; Artinger *et al.*, 1997a).

A more precise mapping of the activin and FGF response elements was hampered by the fact that injected DNA does not integrate into the genome. This causes strong variation from experiment to experiment, probably because of mosaic distribution of the injected DNA (Vize *et al.*, 1991). For the same reason it is also difficult to study the effect on the spatial and temporal expression pattern even when such a response element is identified (see below).

Response elements in *Xenopus* promoters

The best characterised activin response element to date is present in the *Mix.2* promoter (Chen *et al.*, 1996a; Chen *et al.*, 1997). This response element constitutively albeit weakly binds Fast-1, a transcription factor of the Forkhead family. By itself Fast-1 acts neither as a transcription activator or repressor. However, upon activation of the activin pathway, a Smad2/Smad4 complex moves into the nucleus and binds to Fast-1 (Chen *et al.*, 1997). The resulting complex has enhanced affinity for the activin response element and confers transcriptional activation. Several regions in other *Xenopus* genes have also been identified that are able to confer activation in response to activin, including *gsc* (Watabe *et al.*, 1995), *Lim-1* (Rebbert and Dawid,1997), and *Xfkh1* (Friedle *et al.*, 1998; Howell and Hill, 1997). Interestingly, neither the region of *Xbra* that responds to activin nor any of the other *Xenopus* activin response regions contain a consensus binding site for the Fast-1 protein. Furthermore, they do not show any obvious homology to each other. This suggests that there are several mechanisms that can mediate a response to activin.

A putative FGF response element consisting of multiple copies of SRF and ETS factor binding sites has been identified in the *Xegr1* gene (Panitz *et al.*, 1998). Injection of RNA encoding a dominant negative version of Elk-1, an ETS type protein (dnElk-1), suppresses induction of *Xegr1*, while a constitutively active version of SRF (SRF-VP16) stimulates *Xegr1* expression in absence of FGF (Panitz *et al.*, 1998). Interestingly, dnElk-1 has no effect on *Xbra* induction, while SRF-VP16 even suppresses *Xbra* expression (Panitz *et al.*, 1998). This indicates that induction of *Xbra* via FGF is mediated via a different mechanism from that which activates *Xegr1*.

Promoter elements mediating repression

As with the homeodomain binding sites in the *Xbra* promoter, other *Xenopus* promoters also contain binding sites for repressors which have been implicated in restricting their expression domains. For example *Xnr3*, *siamois* and *twin* are all activated by the Wnt pathway via nuclear ß-Catenin on the dorsal side of the embryo. This activation is mediated via several TCF-3 binding sites in the promoters of the respective genes (McKendry *et al.*, 1997; Brannon *et al.*, 1997; Laurent *et al.*, 1997). Interestingly, the same binding sites mediate repression on the ventral site of the embryo (Fan *et al.*, 1998). This was recently elucidated by the demonstration that TCF-3 interacts with the ubiquitous expressed *groucho* repressor and that Groucho and ß-Catenin compete for binding with TCF-3 (Roose *et al.*, 1998).

Gsc, which also interacts with Groucho, is able to negatively autoregulate its own expression by binding to paired type homeodomain sites overlapping with the activin and Wnt response elements in its promoter (Jimenez *et al.*, 1999).

Finally Xvent1 can bind to the *XFkh1* promoter and can mediate BMP-4 induced suppression of the gene (Friedle *et al.*, 1998) and BMP-4 induced repression of the *gsc* gene has been shown to be mediated by binding of Xom/Xvent2 to the activin response element in the *gsc* promoter (Watabe *et al.*, 1995; Trindade *et al.*, in press). This repression of *gsc* and *XFkh1* in the presence of BMP-4 signalling was suggested to be important for restricting these genes to the organiser region (Friedle *et al.*, 1998, Trindade *et al.*, in press)

Studying the spatial and temporal effects of promoter elements in *Xenopus*

Although the above studies suggest that repression is an important mechanism in regulating gene expression, the precise effects of the repressor elements on spatial and temporal aspects of expression have not been addressed. However, if we want to understand how a gene becomes confined to a specific tissue it is important to be able to study these effects.

As stated before, it is not possible to study the precise temporal and spatial expression of promoter fragments by simple injection of these fragments into the one cell embryo. The resulting highly mosaic expression does not allow proper localisation of the expression domain. In addition it has been shown, in at least some cases, that expression from a promoter on plasmid DNA does not reproduce the spatial localisation of the same

promoter fragment integrated into the genome (Kroll and Amaya, 1996). Thus, in order to further study regulation of *Xbra*, I decided to make use of a recently developed method to create transgenic frog embryos, where the exogenous DNA is integrated into the genome during the first cell cycle (Amaya and Kroll, 1996). This method was shown to result in the correct spatial and temporal expression of the cardiac actin and N-tubulin promoters and allows the CMV and cytoskeletal actin promoters to drive ubiquitous expression of reporter genes. (Kroll and Amaya, 1996).

Using this method I demonstrated that the proximal *Xbra* 5' flanking region is sufficient to drive expression of a reporter gene throughout the marginal zone at the early gastrula stage. Interestingly, expression in the notochord is rapidly down-regulated thereafter, suggesting as in the mouse, that expression in the notochord is mediated by an element outside the vicinity of the 5' promoter (Clements *et al.*, 1996). Deletion analysis and the creation of point mutations in putative transcription factor binding sites have identified two distinct repressor elements within this region. Disruption of these elements causes widespread expression of reporter constructs at different stages of gastrulation. My results suggest that the restriction of *Xbra* expression to the marginal zone is achieved predominantly by specific repression in areas where it should not be expressed, rather than specific activation in areas where it is needed. A similar mechanism may be involved in the regulation of other genes in early embryonic development.

3.2. Results

3.2.1. Establishing the transgenic technique

When I started the research for this PhD thesis, the technique developed by Drs Enrique Amaya and Kristin Kroll (Amaya and Kroll, 1996) was not yet published. Generously, Dr Enrique Amaya provided our lab with the protocol for the method in advance. However, the protocol had nevertheless to be adjusted to the circumstances in our laboratory. As establishing and adjusting the technique took a major part in the project for this thesis I will give a brief summary of the results of this process.

Embryos transgenic for a 580 bp cardiac-actin promoter fragment express a reporter consistently in heart and somites

For setting up the technique I used 580 bp of the 5'-flanking cardiac-actin promoter region fused to a GFP reporter gene (kindly provided by Tim Mohun, NIMR, London). The resulting protocol for the generation of transgenic embryos (see Chapter 2) differs only in details from the original publication of Amaya and Kroll (1996). The frequencies of survival and efficiency of transgenesis were similar to those described by Amaya and Kroll (1996). Most importantly, expression of the 580 bp cardiac actin GFP construct was consistently seen in heart and somites in all embryos analysed (Fig. 3.1, n=43, stage 35-42). Only three embryos showed illegitimate expression in individual ectoderm cells, possibly because of unintegrated plasmid DNA (not shown). However, the expression level was subject to strong variation from embryo to embryo. Each individual experiment resulted in expression levels varying from barely visible to very strong. This observation might be

explained by variation in copy number and integration sites in the individual embryos. Although this makes it impossible to draw conclusions about the expression level for a promoter construct from an individual embryo or a small number of embryos, the consistency of spatial expression was promising for proceeding with the identification of regulatory elements in the Xbra promoter.

Methods of detection

The use of GFP as a reporter has the advantage that reporter gene expression can be analysed in several different ways. Fig. 3.1 shows an example of the three methods of detection of a GFP reporter gene being driven by the 580bp cardiac actin promoter in tailbud embryos. A more detailed study of the cardiac-actin promoter was performed by Cooper (1999). Expression of the endogenous gene occurs in somitic muscles and in the heart at this stage (stage 35; Mohun *et al.*, 1984). Fig. 3.1A shows direct visualisation of GFP protein in the somitic muscles under UV light. This detection method has the advantage that expression can be monitored in living embryos. However, the method is not suitable for an analysis of the exact spatial expression, because internal expression cannot be analysed. This is because at early embryonic stages in particular, embryos are opaque and have relatively high levels of autofluorescence in the yolk at a similar emission frequency to GFP.

Fig. 3.1B, C shows transgenic embryos stained for GFP RNA by whole-mount in situ hybridisation. This method is very sensitive and allows analysis of a large number of embryos in a single experiment. In addition stained embryos can be cleared (Fig. 3.1B, C) or sectioned to reveal internal staining. However, unlike direct observation of GFP.

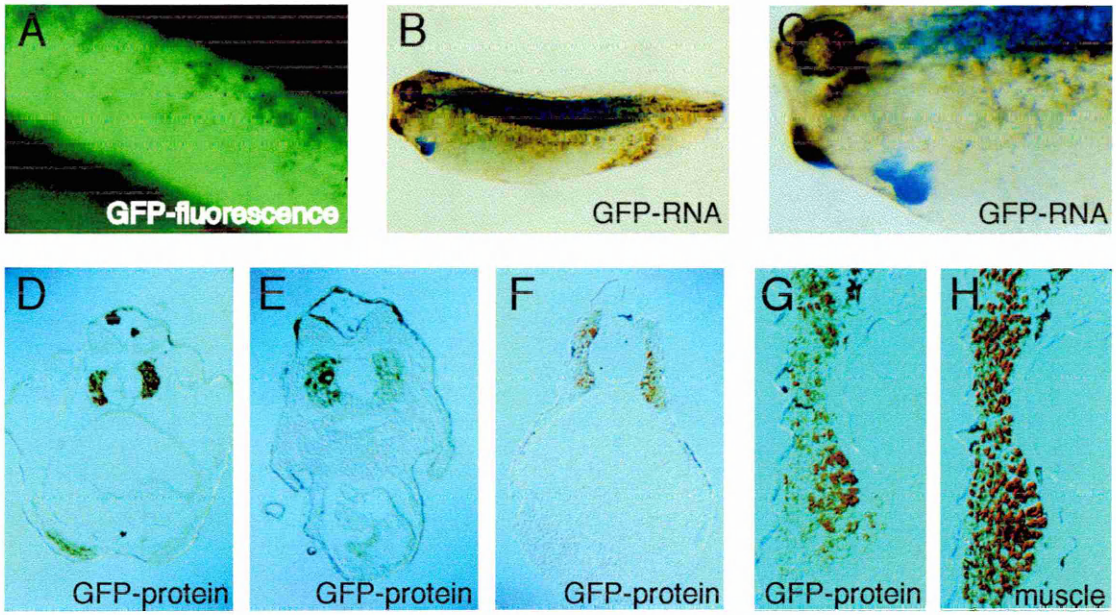


Figure 3.1. Different methods for detection of GFP expression in transgenic cardiac-actin-580-GFP Xenopus embryos.

A. cardiac-actin-GFP expressing muscle tissue of a living stage 35 embryo visualised under UV light. **B, C.** different magnification of a cleared transgenic embryo stage 35 processed with whole-mount *in situ* hybridisation using an antisense GFP probe. **D-G.** 10μm paraffin sections of a stage 40 embryo immuno-stained with a primary antibody against GFP and a secondary antibody fused to HRP for the colour reaction. **D.** anterior section, staining in facial muscle and somites. **E.** section through the heart showing staining in somites and heart. **F.** more posterior section with staining only in somitic tissue. **G.** magnification of F, focused on expression in somites. **H.** same view of a section adjacent to the one in F but immuno-stained using the 12/101 antibody, which recognises an epitope specific for muscle.

fluorescence, embryos have to be fixed and can be analysed at only one stage in development

Detection of GFP protein on sections has the advantage of visualising internal expression domains independent of problems with probe diffusion (Fig. 3.1D-G). By comparing the expression of c-actin-580-GFP with binding of the muscle specific monoclonal antibody 12/101 on adjacent sections (Fig. 3.1G,H), it becomes clear that GFP is expressed in all muscle cells, although the level of expression varies from cell to cell. This effect was seen in two embryos, which both strongly expressed of GFP reporter. However, the small number of embryos analysed with this method does not allow one to draw conclusions as to whether this is a general feature of *Xenopus* transgenesis.

For the following study of the *Xbra* promoter it was found most suitable to analyise expression by whole mount in situ hybridisation. Although expression was also monitored by GFP fluorescence, this has proven particularly difficult for early embryonic stages because of the high autofluorescence of the yolk in the vegetal hemisphere.

3.2.2. Spatial and temporal expression pattern of the Xbra promoter

The *Xbra* promoter used in this study is from the *Xbra2* gene, which is a pseudo allele of the originally identified *Xbra* gene with identical spatial and temporal expression (Latinkic *et al.*, 1997). Preliminary experiments using DNA injection into *Xenopus* embryos have suggested that reporter gene expression driven by 2.1 kp of *Xbra* 5' flanking region is localised to the marginal zone at the early gastrula stage (Latinkic *et al.*, 1997). To

investigate the properties of the 2.1 kb Xbra promoter in more detail, I performed a comparison of reporter gene expression with expression of the endogenous *Xbra* gene at different stages of early embryonic development (Fig. 3.2).

At pre-gastrula stages (stage 9.5-10) promoter activity appeared not to be regulated, and expression was often detectable throughout the embryo, or in segments of it, in both animal and vegetal hemispheres (Fig. 3.2). In some embryos animal and vegetal expression persisted until the early gastrula stage, when GFP transcripts were located either to the right or left side of the embryo and restricted marginal zone expression began in the other half of the embryo. In contrast, specific expression of endogenous *Xbra* began in the dorsal marginal zone and spread to occupy the entire marginal zone by the onset of gastrulation. However, weak ubiquitous expression was also observed for the endogenous *Xbra* gene at stage 9.0 (Fig. 3.2B, see also Panitz *et al.*, 1998; Smith *et al.*, 1991), indicating unregulated expression soon after MBT (see discussion).

After stage 10, expression of the *Xbra* reporter construct was generally confined to the marginal zone, with slightly stronger expression on the dorsal side of the embryo at stage 10.25 (Fig. 3.2A). After stage 10.5, however, expression became down-regulated in the dorsal marginal zone and was absent in the notochord thereafter. Nevertheless, the reporter construct remained active in the circumblastoporal region, and GFP transcripts were detectable in the tailbud at stage 28. This expression pattern resembles that of *Xbra* itself, except that the endogenous gene is expressed additionally in the notochord (Gont *et al.*, 1993).

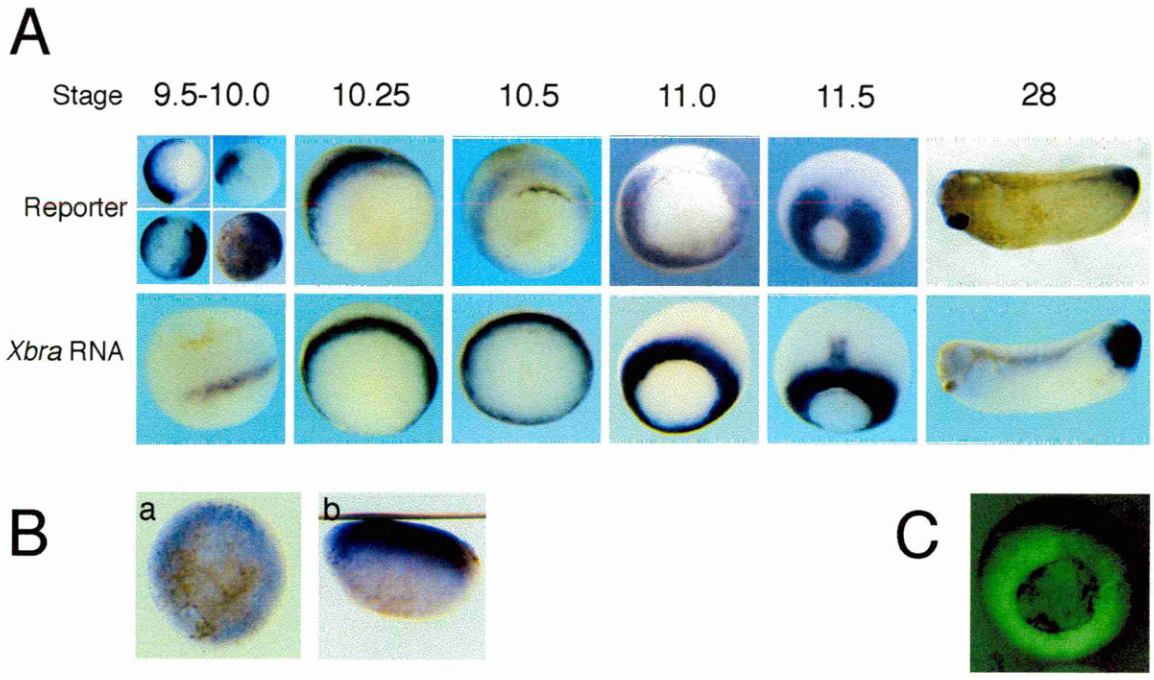


Figure 3.2. A. Time course of the *Xbra-2.1kb-GFP* expression compared to the endogenous Xbra pattern. Dorsal is upwards, except wildtype stage 9.5 (dorsal to the right). The colour reaction to detect reporter expression took considerably longer (12h-48h) compared to the detection of endogenous Xbra RNA expression (5h), indicating a much lower expression).

B. Expression of endogenous Xbra RNA at stage 9.0 visualised in cleared embryos. a: animal pole view, b: animal pole upwards. Nuclear staining indicates newly transcribed zygotic RNA. Staining is specific, because non-transgenic embryos stained for GFP RNA for the same length of time did not show any expression (not shown). Weaker staining in the vegetal pole is probably due to lack of probe diffusion, but see *Xbra* section *in situ* hybridisation in Panitz *et al.* (1998).

C. GFP fluorescence under UV of an embryo at stage 11.0 transgenic for Xbra-4.1kb with GFP fused in frame to the second exon . Note the slightly weaker expression on the dorsal side (top right).

In an attempt to find an element regulating notochord-specific expression of *Xbra*, I also analysed embryos transgenic for a 4.1 kb construct comprising the 2.1 kb 5' flanking region, the first intron and part of the second exon of *Xbra* fused in frame to *GFP*. Expression was markedly enhanced in such embryos such that GFP fluorescence was readily visible under the fluorescence microscope during gastrulation (Fig. 3.2C). The spatial and temporal expression pattern of GFP was, however, identical to that obtained with the 2.1 kb 5' flanking region alone (not shown). Thus expression in the notochord was not detected.

Expression of GFP under the control of 2.1 kb of 5' flanking region during gastrulation was restricted to the marginal zone of approximately 80% (n>150) of transgenic embryos. In the others additional expression was frequently observed in dorsal cells of the animal pole region (not shown)

3.2.3. Deletion study of the *Xbra* promoter

To obtain a crude understanding of the distribution of regulatory elements in the promoter I created transgenic embryos with fragments of the *Xbra* promoter and analysed reporter gene expression during gastrulation.

A fragment containing 970 bp of the 5' flanking *Xbra* region did not show any alteration of the expression pattern during gastrulation (Fig. 3.3B, stage 12, 92%, n=26), but showed consistent illegitimate expression in neural tissues at later stages of development (Xbra-970; Fig. 3.3B, n>25). While expression in neural tissues was also seen

in a proportion of transgenic embryos driving expression from the 2.1 kb 5' flanking region (Xbra-2.1kb; Fig. 3.3A; 20%, n=29), most of those embryos had expression restricted to the tailbud (Fig. 3.3B; 65%; n=29). This suggests the presence of an element specifically suppressing expression in neural tissues situated more than 970 bp upstream of the 5' flanking promoter region.

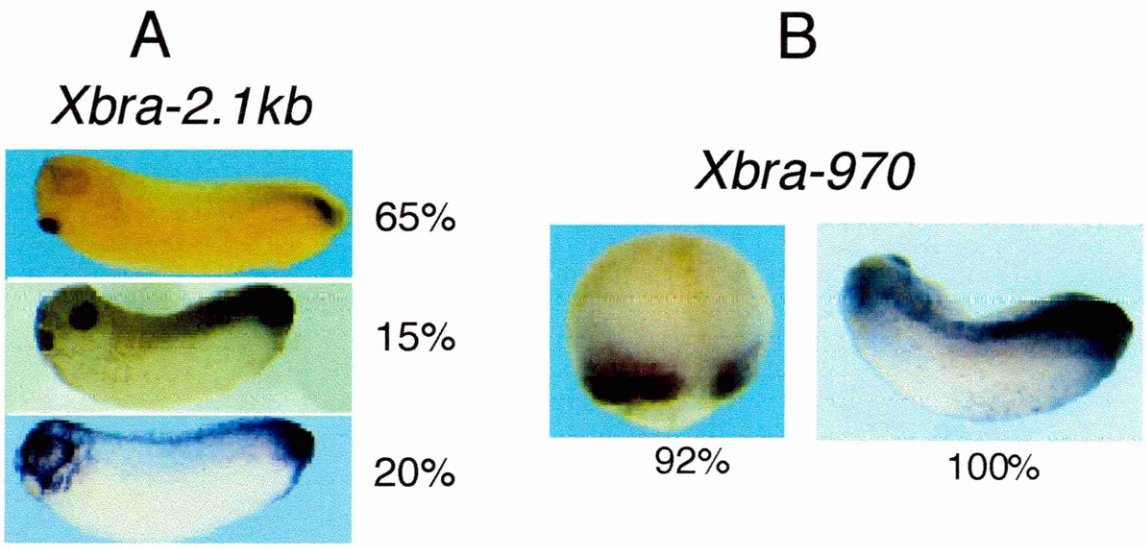


Figure 3.3. *Xbra-970* shows consistent expression in neural tissues at stage 28, but is mesoderm specific during gastrulation.

A. 65% of embryos transgenic for the Xbra-2.1 kb construct show reporter expression restricted to the tailbud, 15% show additional expression in somites and eyes, 20% express in neural tissues and branchial arches (n=29).

B. Reporter expression from the Xbra-970 construct is restricted to the mesoderm in 92% (n=27) of embryos at the end of gastrulation (left panel, stage 12), but shows consistent expression in neural tissues at stage 28 (n>25). Occasional expression in somites does occur in these embryos, but has not been evaluated. Percentages are of expressing embryos.

Fragments containing the first 381 bp proximal to the transcription start site (Xbra-381) were able to recapitulate the marginal zone expression of the 2.1 kb 5' flanking

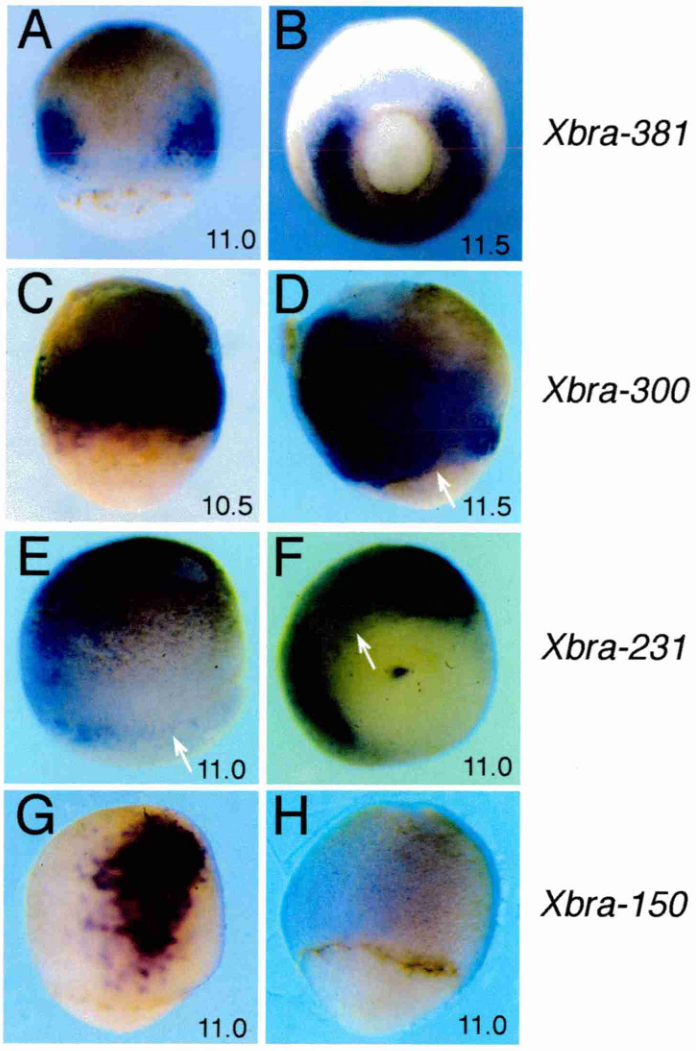


Fig. 3.4. Expression pattern from embryos transgenic for constructs of a deletion series from the Xbra 5' flanking region. A-H: Embryos were analysed during mid-gastrula stages. Representative embryos for each construct are shown. See text for numbers. and explanation.

region (Fig. 3.4A,B; 72%, n=43). However, it is necessary to note that expression was consistently weaker and many embryos additionally had very weak expression in the inner ectoderm layer, only visible in cleared embryos (not shown). In contrast, when a shorter fragment containing 300 bp upstream of the transcription start side was used (Xbra-300) expression was stronger, as judged by the average time of staining. However, the expression borders of mesoderm to endoderm and ectoderm were completely lost (Fig. 3.4C,D; 100%, n>40). Expression extended far into the vegetal pole and strong expression was detected in the inner layer of the ectoderm. In later gastrulation stages, however, there was a gap in the dorsal mesoderm expression, while ectoderm expression was equally distributed (Fig. 3.4D).

Further 5' deletion of the promoter to 231 bp (Xbra-231) resulted in a spatial expression pattern similar to Xbra-300 (Fig. 3.4E, F). However expression was much weaker, and patches of expression were more common than continuous expression. In approximately 50-60% of stained embryos (n=32) there was visibly weaker expression on the dorsal side of the embryo. A 150 bp fragment (Xbra-150) does not appear to show specific expression at all (n>40). Expression varies from a weak continuous expression (Fig. 3.4G) to patches in different parts of the embryo (e.g. Fig 3.4H).

3.2.4. Putative transcription factor binding sites in the proximal *Xbra* promoter

I was most interested in the restriction of *Xbra* expression during gastrulation. The above results showed that a 381 bp proximal promoter region can confer restricted expression to

the mesoderm. Additionally this region contains the smallest promoter fragment that can confer activation by activin and FGF (Latinkic *et al.*, 1997). I therefore decided to look for known transcription factor binding sites in this part of the promoter.

Fig. 3.5A shows a schematic model for regions with prospective promoter elements acting during gastrulation as suggested from the above deletion study. The sequence of the proximal promoter region of the Xbra gene between –482 and +178 is shown in Fig. 3.5B. In the work for this thesis, I have focused on the following putative binding sites (colour coded):

(i) A TATA-box located –25 to 31 bp from the transcription start site, which also overlaps with a putative SRF binding site (see also Fig 5B). Mice with a targeted mutation in SRF never express Brachyury (Arsenian *et al.*, 1998).

(ii) The combination of homeodomain binding sites at –175 to –153, which have been suggested to mediate repression of Xbra at high levels of activin (Latinkic *et al.*, 1997)

(iii) A bipartite binding site for a factor of the δEF1 family at –335 to –300. SIP-1, a recently identified member of this family has been shown to suppress endogenous *Xbra* when overexpressed in the Xenopus embryo (Verschueren *et al.*, 1999).

In addition I noted the presence of a Suppresser of Hairless (Su(H)) binding site (bold), several putative E-box binding sites (bold) and two CCAAT-boxes (bold), which I did not investigate further in this study.

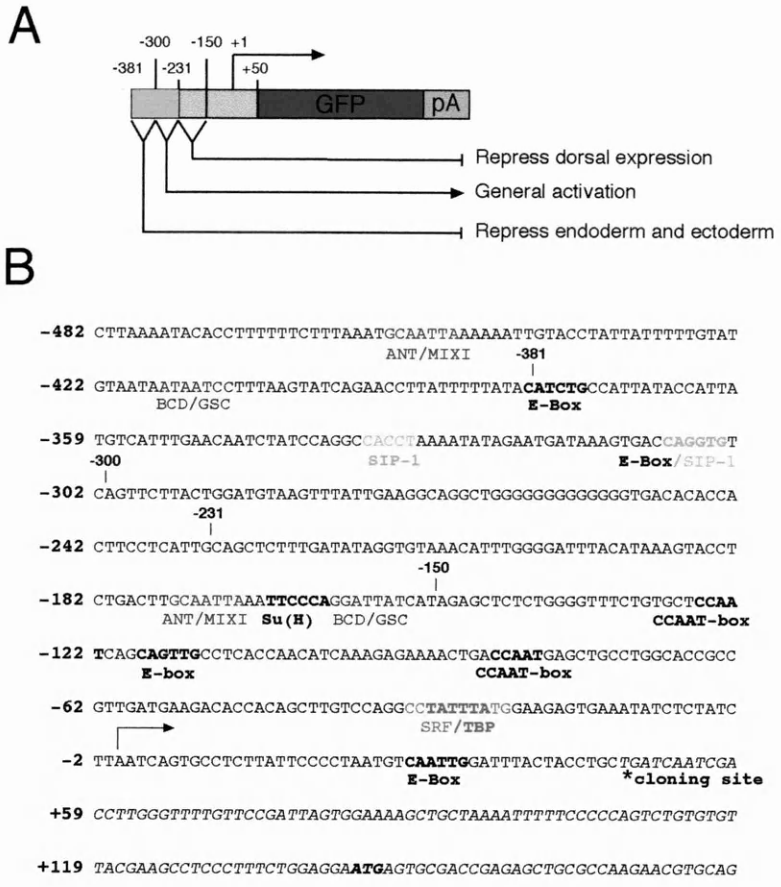


Figure 3.5. Proximal 5' flanking region of the Xbra promoter A. Schematic Model for the location of regulatory elements in the Xbra promoter acting during gastrulation as suggested by the deletion analysis. Elements for FGF and activin responsiveness are expected to be present between –381 and –231 (boxed in pink, Latinkic *et al.*, 1997). **B. Sequence of the proximal Xbra promoter region.** Transcription factor binding sites addressed in this study are colour coded. Additional putative binding sites with regulatory function are represented in bold letters. SIP-1: δEF1 and SIP-1 half site, ANT: antenniapedia type homeobox binding site, BCD: bicoid type homeobox binding site, SRF: serum response factor binding site, Su(H): putative suppressor of hairless binding site. TBP: putative TATA box binding protein binding site. The transcription start site is marked with an arrow. *Cloning site marks the fusion point for the GFP reporter gene (except for Xbra-4.1).

3.2.5. An SRF binding site overlapping with the TATA-box is not necessary for *Xbra* promoter expression

Like many eukaryote promoters *Xbra* has a putative TATA-box located approximately 25 bp upstream of the transcription start site (Fig. 3.6A). Additionally this site appears to overlap with a putative SRF binding site. This fact was of particular interest because targeted mice without functional SRF do not form mesoderm and lack expression of *Brachyury* (Arsenian *et al.*, 1998). The SRF binding in the *Xbra* promoter differs slightly from the consensus ($CC(T/A)_6GG$; Pollock and Treisman, 1990), but a binding assay shows that SRF can specifically bind to this site (B. Latinkic, unpublished observation). In contrast to the SRF binding site in the *Xegr1* gene which mediates response to FGF (Panitz *et al.*, 1998), the ETS factor Elk-1 is not able to form a complex with SRF on this binding site (B. Latinkic, unpublished observation). This is in agreement with studies from Panitz et al (1998) who showed that *Xbra* induction by activin or FGF is not affected by a dominant negative Elk-1.

To investigate the significance of this binding site *in vivo* I created frog embryos transgenic for the 2.1 kb or 381 bp promoter fragments with point mutations in the TATA/SRF site, which would be expected to abolish both binding to SRF and TFIID (Taylor *et al.*, 1989; Leibham *et al.*, 1994) (Fig 3.6A). Surprisingly, these mutations did not affect the spatial or temporal expression of the *Xbra* promoter. Neither in the context of the 2.1 kb promoter fragment (Fig 3.6B, a, b; n>35) nor in the context of the 381 bp fragment (Fig 3.6B, c, d; n>30) did the point mutations have any significant effect on

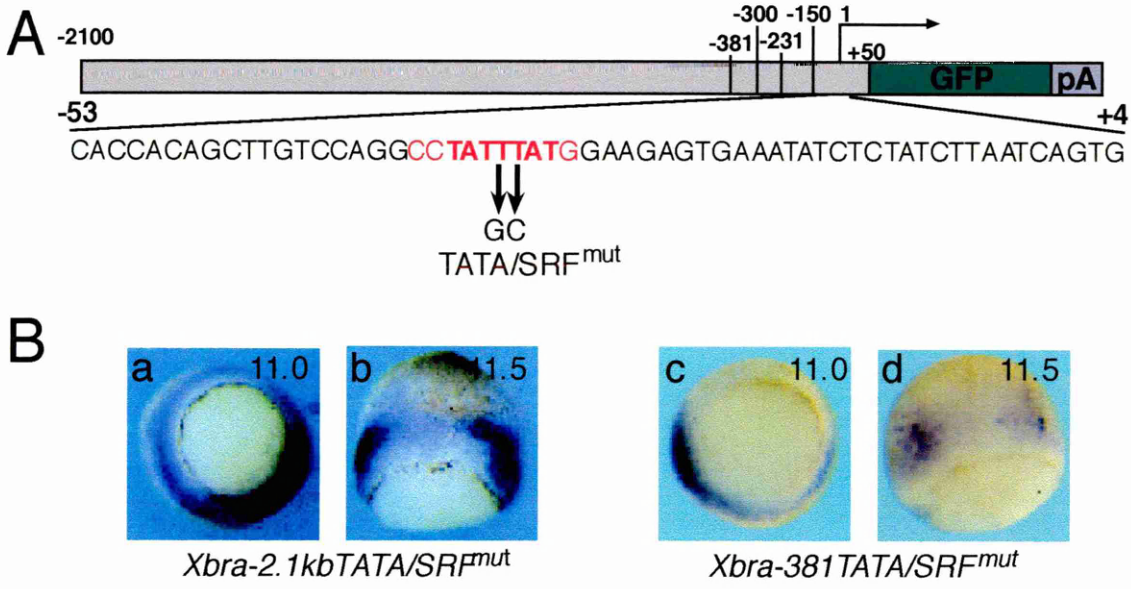


Fig. 3.6. Point mutations disrupting the TATA box and the putative SRF binding site have no effect on the spatial and temporal expression pattern. A. schematic representation with the location of the point mutations. **B.** Expression pattern from Xbra reporter constructs with point mutations in the TATA/SRF binding site. a, b: Point mutations in the context of the 2.1kb Xbra promoter. The embryo in Ba is slightly over-stained revealing weak dorsal expression al stage 11.0. c, d: Point mutations in the context of the 381 bp promoter. See text for numbers and explanation.

reporter expression in the mesoderm. Expression from the 381 bp fragment with point mutations in the TATA/SRF binding site appeared somewhat less uniform than that of the wild-type 381 bp promoter fragment (Fig. 3.4A, B). However, it cannot be ruled out that this is due to natural variation in the transgenic procedure.

These results suggest that neither the SRF binding site nor the TATA-box are necessary for the domain of *Xbra* expression studied here. These promoter elements might, nevertheless, be necessary for correct expression in dorsal mesoderm and

notochord, which could not be addressed in this study, because of lack of the 5' promoter activity in these regions.

3.2.6. A module of two homeobox binding site confers repression of Xbra on the dorsal side of the embryo

The deletion study suggested that an element between –150 and –231 bp might be responsible for suppression of expression on the dorsal side. The most obvious transcription factor binding sites in this region are a pair of homeobox binding sites of the antennapedia type (A) and of the bicoid type (B) class, respectively (Wilson *et al.*, 1996). These binding sites have already been shown to bind Gsc, Mix.1 and Otx-1 *in vitro* and are necessary for the suppression of the 381 bp promoter at high levels of activin (Latinkic *et al.*, 1997).

To investigate whether these binding sites are needed for the correct spatial regulation of the promoter I tested constructs with a point mutation in both or either of the two homeobox sites in transgenic embryos (Fig. 3.7A). The positions of the point mutations are identical to the study of Latinkic *et al.*, 1997) which resulted in release of repression of the Xbra-381 promoter construct at high levels of activin. In the context of the 2.1 kb promoter, point mutations in both these sites have little or no effect on the spatial and temporal pattern of expression (data not shown). There are several other homeodomain binding sites of these types located outside the 381 bp region which could potentially be able to compensate. However, in the context of the 381 bp promoter the effect is striking. At stage 10.5 when the wild-type promoter becomes down-regulated in

the dorsal mesoderm, expression of the 381 bp promoter containing point mutation in A and B is mesoderm specific, but upregulated on the dorsal side (Fig. 3.7B; a). This upregulation was seen in all embryos that showed mesoderm specific expression at this stage (n=15) At stage 11 this dorsal expression expands into the dorsal ectoderm (Fig. 3.7B; b; n>30). Later during gastrulation the expression domain becomes more narrow, stretching from the posterior to the anterior on the dorsal side (Fig. 3.7B; c; n=12). Staining in the dorsal ectoderm and mesoderm is much stronger than expression in the lateral and ventral mesoderm. Extended staining reveals expression in these tissues, but the domain of expression also includes most of the endoderm and all of the ectoderm (not shown).

In order to dissect the effects of the individual binding sides I created transgenic embryos with a reporter being driven by the 381 bp promoter with point mutations either in the antennapedia binding site (A) alone or the bicoid binding site (B) alone. At the mid-gastrula stage a point mutation in the A site caused an expression phenotype similar to combined mutation in A and B, but with less pronounced expression in the dorsal ectoderm (Fig. 3.7B; d, e; n>30). Weak expression was also visible in the endoderm (Fig. 3.7B; e, f and data not shown). At stage 11.5 expression in the dorsal mesoderm and ectoderm receded, however, and there appeared to be equally distributed expression in ectoderm and endoderm (Fig. 3.7B; f; n>30).

Point mutations in the bicoid type homeodomain binding site (B) caused a surprisingly different change in expression. Expression was actually upregulated on the ventral side of the embryo in mesoderm and ectoderm. However, expression was also

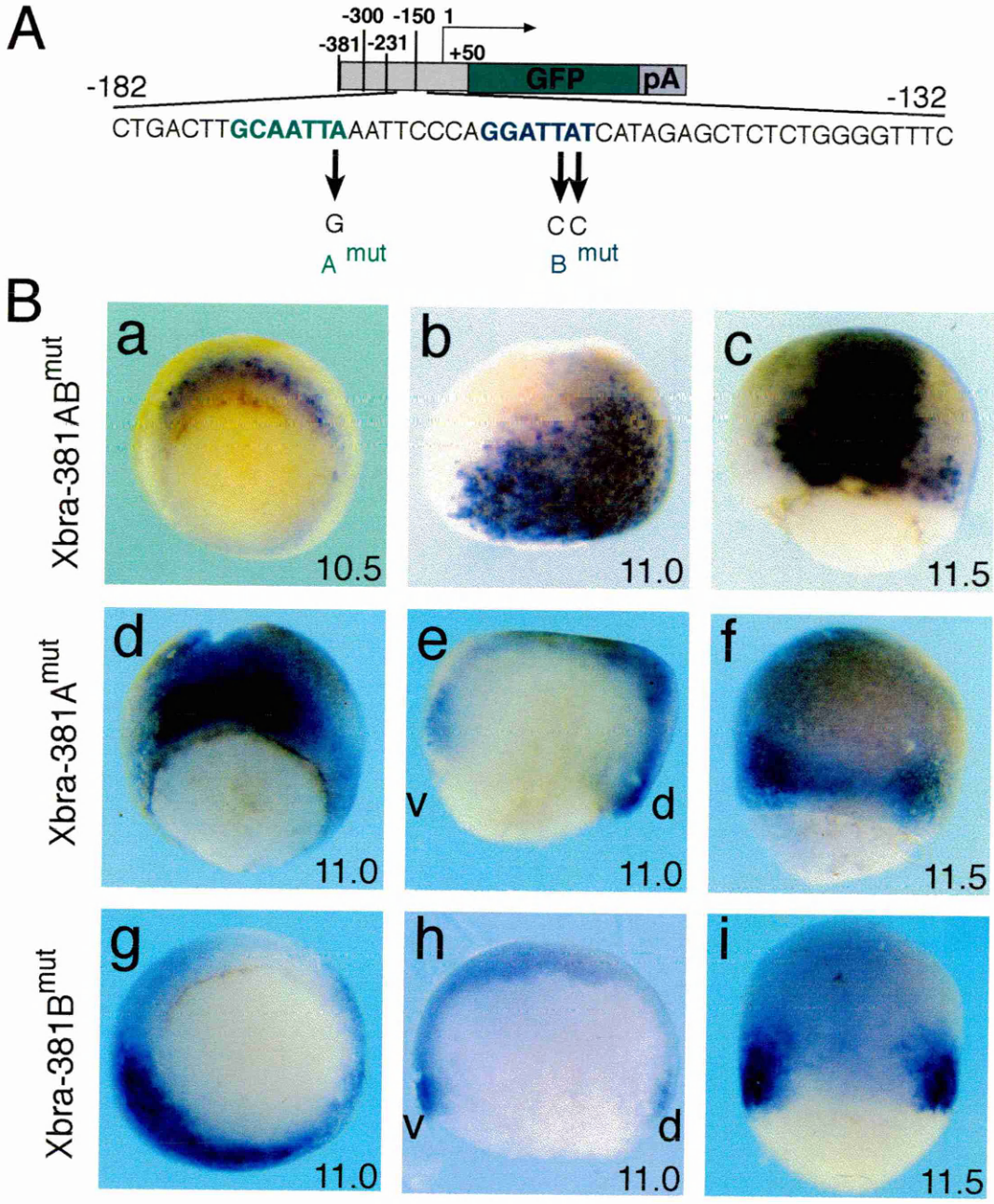


Fig. 3.7. Effects of point mutations disrupting two homeodomain binding sites. A. Schematic representations of the promoter with location of the point mutations. B. Expression pattern of Xbra promoter constructs with point mutations. a, d, g: dorsal to the top. b,e,h: dorsal to the right. c ,f, i: dorsal to the front. Embryos in e and h were cut in half along the ventral dorsal axis to reveal location of internal staining. v: ventral, d: dorsal. The blastocoel of the embryos was collapsed before the in situ procedure to avoid unspecific staining caused by trapping of the probe.

visible in the dorsal mesoderm at stage 11 (Fig 3.7B; g, h; n>30) when there was no expression from the wildtype 381 promoter in this region (Fig 3.4A). At later stages of gastrulation expression in the ectoderm was continuous, while there was a defined gap of expression in the dorsal mesoderm. At no time was expression detected in the endoderm.

These results suggest that both homeodomain binding sites act in concert to repress expression in the dorsal ectoderm at the late gastrulation stage. The A site alone can mediate repression in dorsal ectoderm and dorsal mesoderm at the mid-gastrula stage and mediates also repression in the endoderm. The B site appears to enhance repression of the A site in the ventral ectoderm.

3.2.7. A bipartite δEF-1 binding site is necessary for specifying expression to the mesoderm early in gastrulation

In contrast to the 381 bp fragment, a promoter fragment of 300 bp shows strong expression in endoderm and ectoderm. Thus, I expected that a repressor element in this region might be responsible for establishing the borders between mesoderm and ectoderm and mesoderm and endoderm (Fig. 3.4; C,D). The search for such an element was facilitated by the finding that SIP-1 a novel Smad interacting protein of the δEF1 family, can bind to this region via two CACCT target sequences (Verschueren *et al.*, 1999). This study also showed that SIP-1 can act as a repressor which specifically eliminates endogenous *Xbra* expression when its mRNA is overexpressed in *Xenopus* embryos (Verschueren *et al.*, 1999). In addition, Remacle et al., (1999) showed that the full-length

protein requires both the downstream CACCTG and the upstream CACCT site and that it binds to these sites as a monomer, showing a novel mode of binding where a single protein binds to two distant half sites. In the progress of this study Remacle *et al.* (1999) created four different point mutations in the context of the 2.1 kb Xbra promoter all of which affect high affinity binding of the SIP-1 and δEF1 protein (see Fig. 3.8A). Electro mobility shift assays (EMSAs) showed that mut1, mut2 and mut4 completely abolish binding of the full length SIP-1 protein, while mut3 only decreases the affinity of binding (Remacle et al., 1999). To investigate the effect of these point mutations on the temporal and spatial activity of the *Xbra* promoter in vivo I cloned the promoter fragments containing the point mutations in front of the GFP reporter gene and created transgenic embryos.

A single point mutation in the downstream target half site (CACCTG) in the context of the 2.1 kb promoter (Fig. 3.8A, mut1) results in a complete loss of the mesoderm-ectoderm and mesoderm-endoderm boundaries early in gastrulation (Fig. 3.8B; e, f; 100%, n>30). Misexpression is more extensive on the dorsal side than on the ventral side (Fig. 3.8B; f). Later during gastrulation, the mesoderm-endoderm boundary is re-established, but the embryos show continuous expression in the inner ectoderm layer, readily visible in cleared embryos (Fig. 3.8B; h; 100%, n>30). A different point mutation disrupting the same CACCTG site (mut2) or point mutations disrupting the upstream CACCT site (mut4) in the context of the 2.1 Xbra promoter results in an identical expression phenotype (Fig. 3.8B; i-l, and data not shown). A deletion mutant mut3, which changes the spacing between the target half sites and results in a lower affinity of binding of full length SIP-1 to the promoter (Remacle *et al.*, 1999) causes an intermediate expression phenotype (Fig 3.8B; m-p). Most of the embryos showed some mis-expression

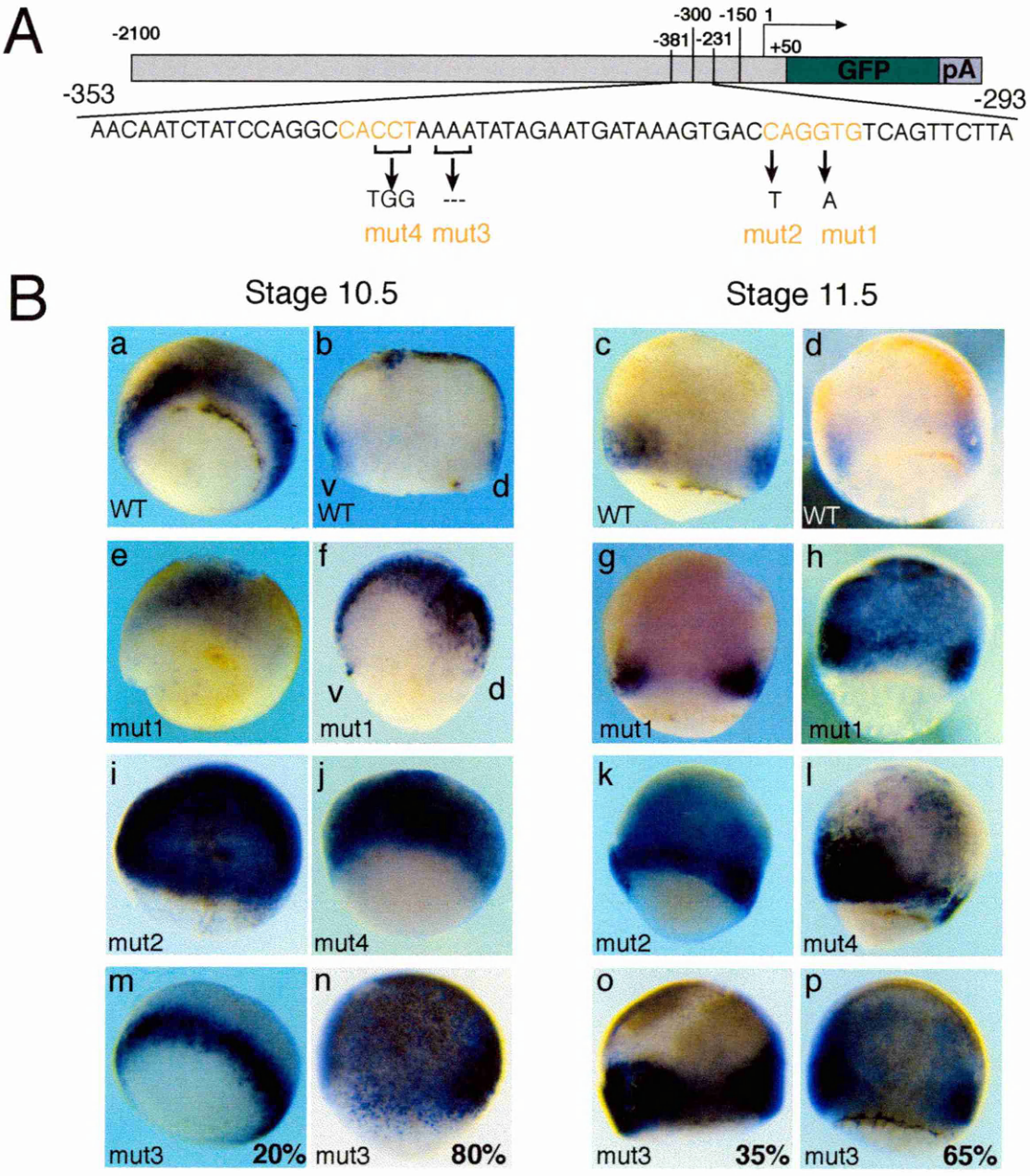


Fig. 3.8. Effects of point mutations disrupting the δEF-1 half sites in context of the 2.1kb promoter. **A.** Schematic representations of the promoter with location of the point mutations. **B.** Expression pattern of Xbra promoter constructs with point mutations. Embryos are located animal pole upward, dorsal to the front, except b, f: dorsal to the right. Embryos in d, h, i, o, p are cleared, embryos in b, f were cut along the ventral dorsal axis to reveal internal staining. Percentages are of expressing embryos. Nomenclature of the point mutations is as in Remacle *et al.* (1999).

(Fig. 3.8B; n, p) but some embryos showed completely normal expression (Fig. 3.8B; m, o; n=35 for stage 10.5; n=41 for stage 11.5).

These results strongly suggest that a protein of the δEF1 family, possibly SIP-1, plays an important role in confining Xbra expression to the mesoderm at the beginning of gastrulation. Preliminary results studying the expression pattern of an *Xenopus* homologue of SIP-1 (XSIP-1) are consistent with this interpretation (Catherine Papin and Leo van Grunvsen, personal communication).

3.3. Discussion

3.3.1. Suitability of the transgenic frog method for promoter studies

In this chapter I have addressed the spatial and temporal regulation of the 5' flanking *Xbra* promoter and have identified two repressor modules, whose integrity is necessary to confine expression to the mesoderm. I have made use of a recently developed sperm nuclear transplantation method, which allows integration of exogenous DNA into the genome and thus analysis of spatial and temporal regulation of promoter constructs in all cells of the embryo (Kroll and Amaya, 1996, this study). To my knowledge this is the first promoter study using this method and I have thus shown that the method is suited to identify promoter elements necessary for spatial and temporal regulation. Kroll and Amaya (1996) have previously used the cardiac-actin and the neuro-tubulin promoter to drive tissue-specific expression of a transgene. In those cases expression was correctly localised in all expressing embryos. I have confirmed this result for a shorter fragment of the

cardiac-actin promoter. Even in the case of the longest *Xbra* promoter construct, variation was much stronger, than was seen with cardiac-actin, such that 15 – 38% of embryos showed additional expression in areas where the endogenous gene is not activated. This expression could be explained by recombination events that may result in disruption of repressor elements. Kroll and Amaya (1997) saw unexpected bands in southern blot analysis of transgenic embryos that indicate recombination events in reporter constructs. However, the promoters used in their study were late tissue specific promoters, which are likely subject to a simpler, mainly positive regulation (Arnone and Davidson, 1997). Disruption of regulatory elements in these cases would result in absence of expression instead of mis-expression, and thus would only decrease the frequency of transgene expression, but not affect the spatial expression.

3.3.2. Unspecific regulation of the *Xbra* promoter before gastrulation

Before gastrulation the *Xbra* promoter is not specifically regulated and relatively strong ubiquitous expression can be detected in either the whole embryo, or part of it. This ubiquitous expression before gastrulation was observed with all *Xbra* promoter constructs tested, including the constructs with point mutations in repressor modules (not shown). Interestingly, weak ubiquitous and thus enhancer independent expression is also seen for the endogenous Xbra gene (this study, Panitz *et al.*, 1998), albeit at a slightly earlier stage.

The genomic DNA in *Xenopus* undergoes a change in chromatin structure between the mid-blastula transition and the end of gastrulation (Dimitrov *et al.*, 1993, reviewed in Patterton and Wolffe, 1996). During this transition the linker histone H1

replaces the B4 protein. This change in chromatin structure has been implicated in the lineage restriction and repression of several genes between MBT and the end of gastrulation (Rupp and Weintraub, 1991; Wolffe, 1989, Bouvet *et al.*, 1994) and causes the loss of mesodermal competence at early gastrulation in *Xenopus* (Steinbach *et al.*, 1997). A transition from enhancer independent to enhancer dependent expression of transgenes occurs also in the mouse around the onset of zygotic transcription at the two cell stage (Martinez-Salas *et al.*, 1989).

Integration of exogenous DNA occurs at average in 4-8 different sites in the genome per transgenic and does not only happen in the first cell cycle, but also in subsequent cell cycles (Kroll and Amaya, 1996). Thus the prolonged ubiquitous expression from the *Xbra* promoter in part of the embryo could be explained by integration events at a site in the genome where the transition from unregulated to regulated expression occurs later than at the site of the endogenous *Xbra* gene.

3.3.3. Absence of an element responsible for notochord expression

Unlike the endogenous *Xbra* RNA, reporter gene expression driven by 2.1 kb of 5' flanking sequence becomes down-regulated on the dorsal side between stage 10.5 and stage 11.0 and is never expressed in the notochord. I have shown that a combination of two homeodomain binding sites is responsible for this down-regulation of expression. Point mutations that disrupt both the sites show strong expression in dorsal ectoderm and mesoderm, including the presumptive notochord domain.

The *Xbra* promoter fragments used in this study lack elements responsible for notochordal expression. In contrast, in the ascidian Ciona intestinalis the promoter of a *Brachyury* homologue (CiBra) was identified that drove expression only in the notochord, but not in other mesoderm derivatives (Corbo *et al.*, 1997). Corbo *et al.* (1998) have shown that two binding sites for suppresser of Hairless Su(H) sites are necessary for notochord expression. Interestingly, in the *Xbra* promoter a single putative Su(H) is located between the two homeobox sites responsible for dorsal repression (Fig. 3.5B), leading to the possibility that a different element responsible for notochord expression could interact with this site to relieve dorsal repression. However, regulation of notochord expression in *Xenopus* appears to be more complex than in ascidians. The CiBra promoter does not drive specific expression in the notochord in *Xenopus* and cannot rescue notochord expression when placed upstream of a 970 bp 5' flanking *Xbra* sequence (not shown).

3.3.4. The *Xbra* promoter can compensate for disruption of the putative TATA box

The *Xbra* promoter contains a TATA box 25-31 bp upstream of the mapped single transcription start site (Latinkic *et al.*, 1997). The start site is surrounded by a sequence that fits the consensus of an initiation region (INR) of Py Py $A_{(+1)}$ N (T/A) Py Py (Javahery *et al.*, 1994; where Py=pyrimidines) in 6 out of the seven sites. Closer examination of the promoter revealed an alternative TATA box 16bp downstream of the original TATA box (Fig. 3.9). This by itself would be too close to the original INR to confer efficient transcription initiation. However, there is also another consensus site for an alternative INR 14 bp downstream of the original transcription start site, which could work together

with the alternative TATA box at the necessary distance of 25-30 bp (Fig. 3.9). Although I did not investigate this possibility further, its presence could offer an explanation why the disruption of the TATA box did not have an effect on spatial or temporal expression. Previous studies have not shown any evidence for an alternative start site as only one band was observer in primer extension experiments (V. Cunliffe and J. Smith unpublished observation) and RNAse protection assays (Latinkic *et al.*, 1997). It is unclear at present whether the occurrence of an alternative TATA box and INR is just coincidence or whether it has a physiological relevance for promoter regulation not provided by the proximal 5' flanking region. This might include regulation of expression in the notochord.

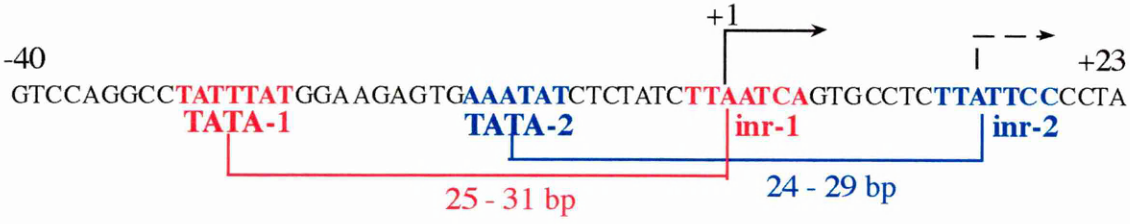


Fig. 3.9. The Xbra promoter contains an alternative TATA-box that could work together with an alternative initiation region. Primary TATA box and primary initiation region (inr-1) are depicted in red. Putative secondary TATA box and secondary initiation region (inr-2) are depict in blue. Both pairs have the optimal distance (25-30 bp) from each other.

Disruption of the TATA box disrupted also a putative SRF binding site. SRF is necessary for mesoderm formation in mouse, and mice lacking functional SRF protein never express *Brachyury* (Arsenian *et al.*, 1998). My results indicate that if the same situation applies for the frog then SRF regulation of *Xbra* is not direct, but an upstream

situation applies for the frog then SRF regulation of *Xbra* is not direct, but an upstream event of mesoderm formation.

3.3.5. Homeodomain binding sites necessary for dorsal repression

Disruption of two homeodomain binding sites located in the proximal *Xbra* promoter results in strong upregulation of expression in dorsal mesoderm and ectoderm, with additional weak expression in the endoderm and the rest of the ectoderm. The effect of this double mutation appears to represent the sum of the effects of the single mutations, with the exception of expression at late gastrula stages, where disruption of neither of the individual sites caused the strong upregulation in the dorsal ectoderm seen in the double mutation.

Several studies have implicated the homeobox gene *gsc* in negative regulation of *Xbra* (Artinger *et al.*, 1997b; Latinkic and Smith, 1999b; Latinkic *et al.*, 1997). *gsc* is expressed in the dorsal mesendoderm at the beginning of gastrulation and in the anterior mesoderm later in development (Steinbeisser and De Robertis, 1993). Although part of this domain is included in the upregulation of expression in constructs that have both homeodomain sites disrupted, the main area of misexpression in the dorsal ectoderm is not consistent with *gsc* expression. Furthermore Gsc protein would be expected to bind to the bicoid type binding site (Wilson *et al.*, 1993a). Disruption of this site, however, causes upregulation in the ventral ectoderm and not in the dorsal mesoderm or endoderm. These results suggest that while Gsc can suppress *Xbra* promoter activity in an overexpression system, other homeodomain proteins appear to regulate *Xbra* expression via this site *in*

vivo. This is in agreement with a recent study of Papin and Smith (in press) which suggest that proteins different from Gsc are responsible for mediating the repression of *Xbra* at high doses of activin.

No known bicoid or antennapedia type homeodomain protein which functions as a repressor fits the expression pattern caused by disrupting the binding sites. Because of the complexity of the expression phenotype when the sites are mutated it is possible that different homeodomain proteins bind to this site in different tissues and/or at different stages of development. Catherine Papin in Dr Smith's laboratory is currently undertaking a one-hybrid screen with this site to search for candidate homeodomain proteins.

3.3.6. A protein of the δEF1 family is likely to confine Xbra expression to the mesoderm

Vertebrate members of the δEF1 family are large zinc finger/homeodomain-like DNA binding proteins that act as transcriptional repressors (Sekido *et al.*, 1997). In a recent study we have shown that members of this family bind DNA as a monomer at a bipartite binding site, where high affinity binding requires to contain the sequence CACCTG and the other site (Remacle *et al.*, 1999). Here I have shown that any point mutation that disrupts high affinity binding of δEF1 family members to the *Xbra* promoter interferes with the restriction of expression to the mesoderm at the beginning of gastrulation and results in widespread mis-expression in ectoderm and endoderm. Subsequently during mid-gastrula

stages, expression becomes confined to a ring in the marginal zone mesoderm, but it continues to be mis-expressed in the inner layer of the ectoderm.

Both phenotypes are consistent for all point mutations disrupting the newly defined δEF1 family binding site (Remacle *et al.*, 1999). However one member of this family, SIP-1 (Verschueren *et al.*, 1999), is of particular interest because it interacts with activated Smad proteins and has been shown to interfere with endogenous *Xbra* expression when overexpressed in the embryo (Verschueren *et al.*, 1999). Members of the dEF1 family are thought to exert their repressive effect in two ways. Their binding site coincides with an E-box binding site, where activators such as δEF3 can bind, thus they could compete with binding of activators. In addition they are also active repressor with repressor domains located at the N-terminal and C-terminal end of the protein (Sekido *et al.*, 1997). SIP-1 is unique in this family in the sense that it has an additional domain that has been show to interact with activated receptor specific Smad proteins, such as Smad1, Smad2 and Smad3 (Verschueren *et al.*, 1999). One could hypothesise a model where SIP-1 is bound to its binding site, but changes its conformation and lifts off the DNA when

Fig. 3.10. Hypothetical Model for a relief of SIP-1 mediated repression via an activated Smad molecule. SIP-1 is tightly bound to DNA via its bipartite DNA binding domain. Activated Smad molecules can interact with SIP-1 via its Smad interaction domain. One could speculate that such an interaction changes the conformation of SIP and the molecule lifts off the DNA.

associated with an activated receptor regulated Smad molecule (Fig. 3.10). This model is currently under investigation.

3.3.7. Repression as an important mechanism to achieve region specific expression in early embryonic development

An interesting problem in early embryonic development is how a specific tissue acquires its spatial identity. This can be restated to the question of how transcription factors that specify a certain tissue are localised to it. Here I have shown that in the case of *Xbra*, which is required for specification of the mesoderm, this is achieved by a rather general activation combined with repression in places where it should not be expressed. A similar mode of action was suggested for the *Xfkh1* gene (Friedle *et al.*, 1998), which is expressed in the organiser region. *Xfkh1* is activated by Smad2 via activin signalling through an element in intron-1 (Howell and Hill, 1997) and is repressed by the *Xvent1* gene, which is active in ectoderm and mesoderm except in the organiser region (Gawantka *et al.*, 1995). Repression is mediated by a homeodomain binding site in the 5' flanking region of the *Xfkh1* gene (Friedle *et al.*, 1998). However, the actual spatial effect of disrupting the element was not analysed in their studies.

In other promoters specific activation of a gene utilises switching a repressor element into an activator element as is observed with the TCF-3 binding site in the siamois and *Xnr-3* promoter. When complexed with Groucho TCF-3 represses the promoter on the ventral site (Roose *et al.*, 1998), but activation occurs via the same element when ß-Catenin binds to TCF-3 on the dorsal side (Fan *et al.*, 1998). Although this relatively simple

mechanism might work to distinguish between dorsal and ventral domains over a relatively large area, it might not be sufficient to set up very confined regions such as the prospective mesoderm. My results suggest that *Xbra* regulation is very complex with different activator and repressor modules acting in concert, but this might precisely reflect the complex information that is needed to define mesoderm. Complex promoters involving multiple activator and repressor elements in genes important for defining spatial identity have already been described in other systems such as in fly and sea urchin (reviewed in Arnone and Davidson, 1997). It can be expected that vertebrate promoters have a similar degree of complexity.

3.3.8. Downstream of Xbra

Interestingly, a single T-box binding site in front of a minimal promoter results in specific expression of a reporter in the mesoderm during gastrulation (Casey *et al.*, 1999). Such T-box sites are important for the mesodermal expression of down-stream targets of Xbra, such as eFGF (Casey *et al.*, 1998a) and the Bix family (Casey *et al.*, 1999; Tada *et al.*, 1998). This shows that once the regional identity of a tissue is defined by a transcription factor, regulatory elements could be very simple. In addition it stresses the importance of *Xbra* and possibly other T-box genes in defining the regional identity of the mesoderm. Now that we have a tool to address the precise effect of binding sites on spatial expression in *Xenopus*, we can go one step further to address which information is needed to tell a confined tissue to become mesoderm. Further studies of the proteins that regulate the Xbra promoter, such as SIP-1, will help to provide some answers to this question.

4. FUNCTIONAL ASPECTS OF THE XBRA PROTEIN

When I set out to do the work for this thesis it was to learn about both, the regulation and functional aspects of *Xenopus Brachyury*. However, in the course of the study it turned out that my main attention had to be focused on the regulation of *Xbra*. Nevertheless I did address some functional aspects of the protein and would like to present my results in this Chapter. The reader may be warned that this is not a completed project and some of the results are only preliminary, but they might open the door for further investigation. If the reader completes this Chapter with the feeling that there is still much to learn about the function of this fascinating protein, then this Chapter has achieved its purpose.

As has been discussed in Chapter 1.3., Xbra is a transcription factor and activates several types of target genes important for mesoderm formation. In this study I have been particularly interested in three main aspects of the function of Xbra protein.

i) Which part of the Xbra protein is responsible for its localisation to the nucleus; and is this nuclear localisation regulated, and if so, how?
ii) Does Xbra function as a monomer or a dimer?
iii) Is the Xbra protein phosphorylated and if so, does this phosphorylation play a role in the functional regulation of Xbra protein?

4.1. Introduction

Evidence for Brachyury acting as a dimer

Brachyury is the founder member of the T-box transcription factor family. The T-domain is the DNA binding domain of the protein and was initially shown by nucleotide selection from a random pool of oligonucleotides to bind a palindromic sequence of 20 bp (TTTCACACCTAGGTGTGAAA; Kispert and Hermann, 1993). X-ray crystallographic analysis showed that the Xbra T-domain binds the palindromic sequence as a dimer. The dimer forms a large arc spanning the DNA which allows it to contact the whole region of the 20 bp DNA recognition sequence without bending the DNA (Fig. 4.1; Muller and Herrmann, 1997).

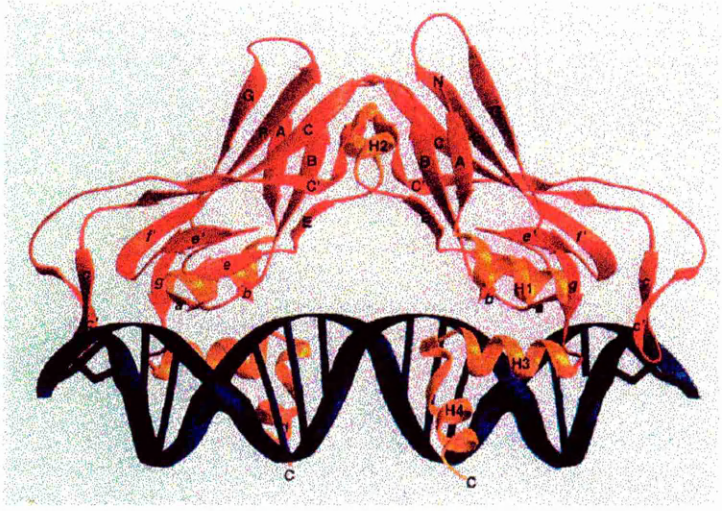


Fig. 4.1. Ribbon diagram of the T-domain dimer bound to DNA. Depicted are residues 39-221 of both monomers (strands and loops: red; helices: yellow; DNA: blue). Taken from Muller and Herrmann (1997).

The dimer interface is weak, which is consistent with the observation that the T-domain by itself is present as a monomer in solution (Steve Smerdon and Jim Smith, unpublished observation).

Although in the absence of DNA the T-domain exists as a monomer, it is possible that the full-length protein has domains that support dimerisation in solution. Evidence that Brachyury might exist as a dimer came from experiments employing a two-hybrid test (Fig. 4.2; kindly provided by Frank Conlon, NIMR, London). The two-hybrid test was performed in the yeast *S. cerevisiae* using a *LacZ* reporter gene downstream of a promoter construct containing several binding sites for the GAL4 transcription factor. The experiments suggested that a *Xbra* construct without a transcription activation domain is able interact with a construct that contains a transcription activation domain but lacks the ability to bind DNA (Fig.4.2).

The domain structure of Xbra is shown in Fig. 4.3. The T-domain, which is responsible for DNA binding, is located in the N-terminal half of the protein (Amino acid 1-226). The transcription activation domain has been mapped to the C-terminal third of the protein (amino acids 303-387; Conlon *et al.*, 1996). The construct used in the two hybrid test that lacked the transcription activation domain (XbraΔ303) still contained a linker region connecting the DNA binding domain with the activation domain. It is thus possible that peptide sequences necessary for homo-dimerisation in solution are present within this region. It should also be noted here that while only a single activation domain was found in the zebrafish and *Xenopus* homologues of Brachyury (Conlon *et al.*, 1996), a previous report claimed the mouse Brachyury

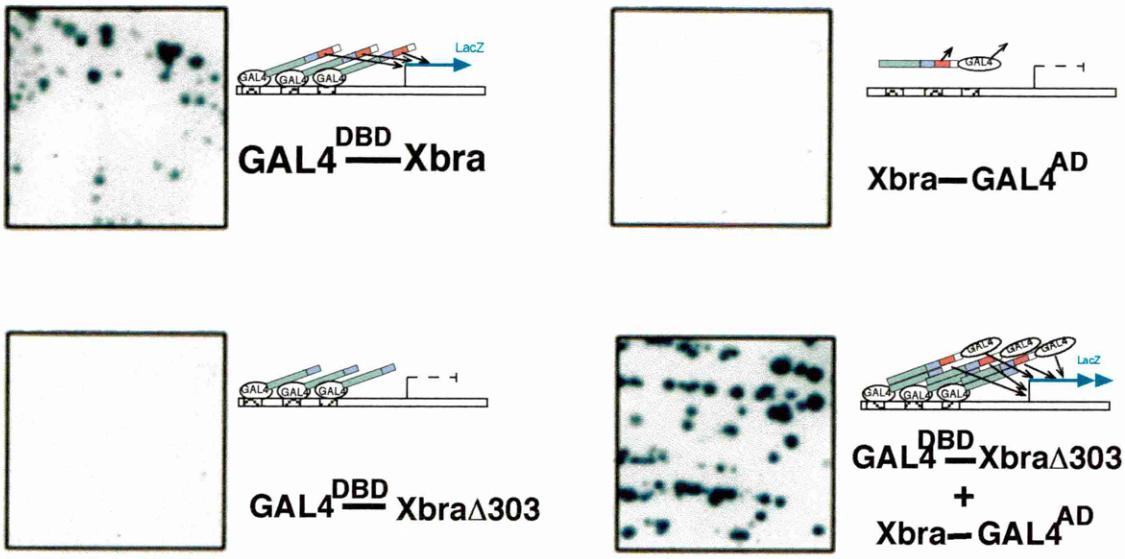
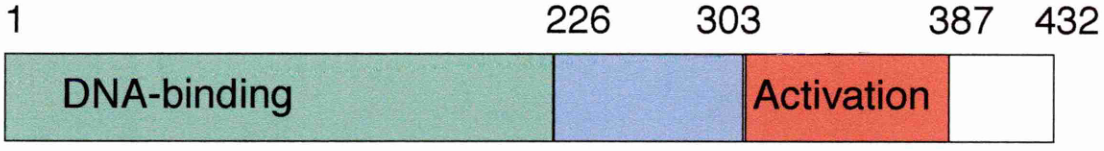


Fig. 4.2. Two hybrid test for the dimerisation of Xbra in S. cerivisae. A yeast strain is carrying a LacZ reporter downstream of a minimal promoter and three UAS GAL4 respond elements. **A.** Xbra fused to a GAL4 DNA-binding domain activates LacZ, because of its own activation domain. **B.** Xbra without its c-terminal region does not show activity, even when DNA binding to the promoter is conferred by the GAL4 DNA-binding domain. **C.** Xbra without the GAL4 binding domain can also not activate the promoter, even when fused to the GAL4 activation domain. **D.** When the strain is transformed with both the constructs in B and C strong activation of the reporter is observed, suggesting that Xbra can interact with its own protein.



4.3. Domain structure of the Xbra protein. The DNA-binding domain is located to the N-terminal half of the protein, while the activation domain is was identified to a region in the C-terminal portion of the protein. Between the activation domain and the DNA-binding domain is a linker region, without clearly specified function.

protein (T) is more complex, with two activator and two repressor domains (Kispert *et al.*, 1995a). This would place an additional activation domain and a repressor domain into what is the linker region of Xbra (Kispert *et al.*, 1995a). The functional significance of such an arrangement has not been addressed. An explanation, however, could be that the linker region contains interaction sites for other proteins, which may also be involved in the dimerisation of Brachyury.

What determines the nuclear localisation of Brachyury?

Brachyury is a transcription factor and thus needs to be in the nucleus of the cell to exert its function. All proteins are synthesised in the cytoplasm and have to enter the nucleus via the nuclear pore complex (NPC), a complex that spans the double lipid bilayer of the nuclear envelope and consists of over 100 different polypeptides (reviewed in Davis, 1995). Molecules of up 50 kDa may pass the NPC by diffusion. Molecules of larger size need facilitated, energy dependent transport (Jans and Hubner, 1996, Gorlich, 1997). However, even small proteins, such as HMG14 and HMG17 (<10kd), require nuclear localisation signals for efficient targeting to the nucleus (Breeuwer and Goldfarb, 1990; Hock *et al.*, 1998). Import into the nucleus can be conferred by several distinct import signals. The best characterised signal is the 'classical' nuclear localisation signal (NLS), which consists of one or more clusters of basic amino acids (Dingwall and Laskey, 1991). This pathway is relatively well characterised and requires the recognition of the NLS by the import receptor importin (Gorlich *et al.*, 1995; Imamoto *et al.*, 1995). GTP hydrolysis by the GTPase Ran/TC4 is necessary for energy dependent transport into the nucleus (Melchior *et al.*, 1993; Moore and Blobel, 1993)

The second known pathway employs a different import receptor, transportin (Pollard *et al.*, 1996), which recognises a 38-residue M9 domain, present in the hnRNPA and hnRNPB proteins (Siomi and Dreyfuss, 1995). NLS and M9 containing proteins do not compete with each other for import into the nucleus (Pollard *et al.*, 1996). The import of U snRNPs (U-rich small nuclear ribonucleoproteins) defines another pathway into the nucleus (Fischer *et al.*, 1991; Michaud and Goldfarb, 1991). It is likely that that in addition several other pathways exist, which have not yet been characterised.

As expected, Brachyury is localised in the nucleus in all cells where it is endogenously expressed (Schulte-Merker *et al.*, 1992; Cunliffe and Smith, 1994; Kispert and Herrmann, 1994). However, neither an obvious basic region (classical NLS) nor any homology to the M9 domain, is present in the Brachyury protein of frog, chicken, zebrafish or mouse (Smith *et al.*, 1991; Schulte-Merker *et al.*, 1992; Knezevic *et al.*, 1997a; Herrmann *et al.*, 1990). Kispert *et al.* (1995), who studied the nuclear localisation of several N-terminal and C-terminal deletions of the mouse Brachyury protein inferred the presence of several complex NLSs between residues 137 and 320. However, those signals are poorly characterised and this issue has not been addressed for Brachyury gene products in other species or for any other T-box protein. Thus it would be interesting to find out how these transcription factors are localised to the nucleus and whether this localisation is regulated in any way during embryonic development.

Is Xbra regulated at the post-transcriptional level?

The question of whether the function of the Brachyury protein is regulated at the post-translational level has not yet been addressed. However, the possibility is not unlikely, because experiments in mouse and *Xenopus* suggest that the quantity of Brachyury in a certain tissue should be tightly controlled (Stott *et al.*, 1993; Cunliffe and Smith, 1994; O'Reilly *et al.*, 1995). Thus ectopic expression of *Xbra* induces different kinds of mesoderm when overexpressed in the animal pole: low concentrations of Xbra induce ventral vesicles, medium concentrations induce smooth muscle and high concentrations induce skeletal muscle. These changes occur over two fold increases in the amounts of Xbra expressed (O'Reilly *et al.*, 1995, Tada *et al.*, 1997). In mouse different levels of a Brachyury transgene can determine to what extent the tail phenotype of +/T mice is rescued (Stott *et al.*, 1993).

The need for tight regulation is also apparent from by the rapid down-regulation of Brachyury protein in the involuting mesoderm following the down-regulation of the RNA (Schulte-Merker *et al.*, 1992, Cunliffe and Smith, 1994, Kispert and Herrmann, 1994). However, such a rapid turnover of the protein would not be necessary (and would in fact be uneconomic) in the presumptive notochord and in the tailbud, where the protein is required for a much longer time. A prolonged life of the protein in the notochord is suggested by the fact that Xbra protein can be detected in the *Xenopus* notochord until tadpole stages, when the *Xbra* RNA is no longer present (Brenda Price and Jim Smith, unpublished observation).

For many proteins, degradation is mediated by specific targeting to the proteasome system. The best known pathway involves phosphorylation-dependent

destruction of a wide array of regulatory proteins via the ubiquitination pathway (reviewed in Bonifacino and Weissman, 1998; Elledge and Harper, 1998). Examples of regulatory proteins degraded by this pathways are IκB and ß-Catenin (Orford *et al.*, 1997; Winston *et al.*, 1999). IκBα is normally associated with NF-κB, to obscure its nuclear localisation signal (Baeuerle and Baltimore, 1988). In response to TNFα and other signals, IκBα is phosphorylated on two serine residues near its N-terminus (Finco *et al.*, 1994; Beg *et al.*, 1993), triggering its rapid destruction by the ubiquitin mediated pathway (Chen *et al.*, 1996b; Scherer *et al.*, 1995; Winston *et al.*, 1999). This allows NF-κB to enter the nucleus and activate target genes (reviewed in Hochstrasser, 1996).

The product of the immediately early gene *c-fos* is also degraded by targeting to the proteasome (Salvat *et al.*, 1998). However, in contrast to the proteins discussed in the above section, phosphorylation of two serines (Ser362 and Ser374) stabilises the C-fos protein by protecting it from degradation (Salvat *et al.*, 1998). This phosphorylation was shown to be mediated by the MAP kinase pathway and is necessary and sufficient for the stabilisation of C-fos in response to the proto-oncogene c-mos (Okazaki and Sagata, 1995). C-mos is a serine/threonine kinase which controls the meiotic cell cycle in vertebrate oocytes (Sagata, 1997; Sagata *et al.*, 1988).

This mode of stabilisation is of particular interest concerning the possible regulation of Xbra protein. *Xbra* is co-expressed with *eFGF* in the marginal zone and in the presumptive notochord, and both maintain each other's expression via an indirect autocatalytic loop (see Chapter 1.3). eFGF signals via the MAP kinase pathway. Like *Xbra*, *eFGF* is not expressed in the involuting mesoderm, where Xbra protein is rapidly

down-regulated. It could thus be hypothesised that eFGF not only regulates *Xbra* on a transcription level, but also stabilises its protein by phosphorylation via the MAP kinase pathway. Evidence for such a possibility will be presented in section 4.2.6.

4.2. Results

4.2.1. N-terminal and C-terminal deletion series of the Xbra protein

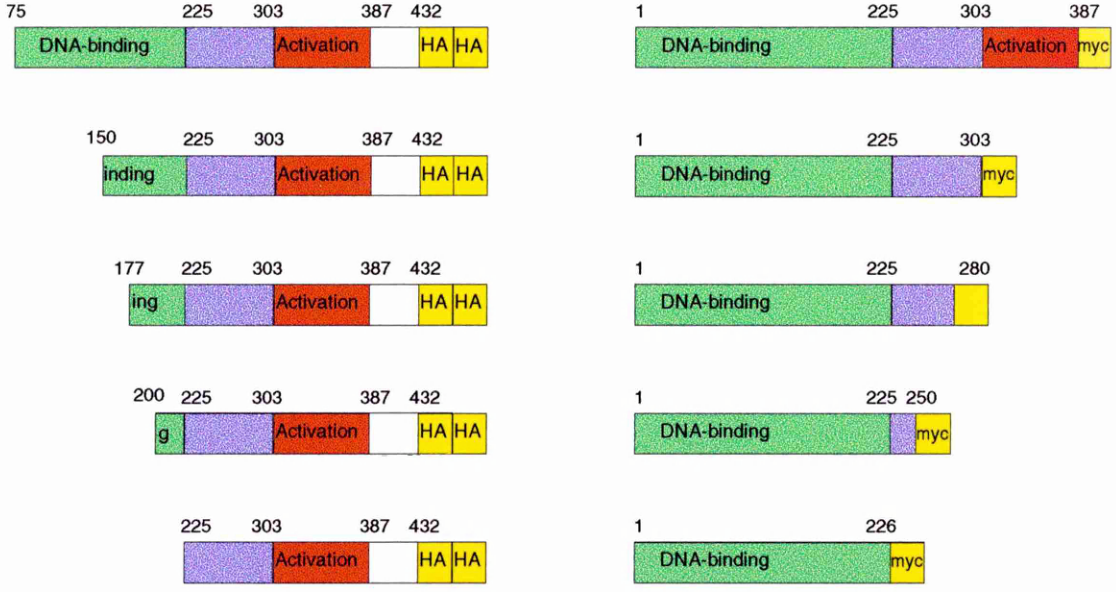


Fig. 4.4. Series of N-terminal and C-terminal deletions of the Xbra protein. N-terminal deletions were fused to two HA tags. C-terminal deletions were fused to a single myc tag

To study functional aspects of the Xbra protein I constructed several tagged N-terminal and C-terminal deletions of the protein (Fig. 4.4). N-terminal deletions were cloned into a vector containing two HA tags, while C-terminal deletions were cloned into a vector containing a single myc tag. The reason for using different tags for C-terminal and N-

terminal deletion constructs was to be able to allow me to contact two hybrid experiments investigating the interaction of constructs lacking the DNA binding with constructs that bind DNA but lack transcriptional activation.

The integrity of the constructs was confirmed by automated sequencing and the stability of the construct was tested by injection into embryos followed by immunoblotting of animal pole extracts at stage 9 with antibody against the HA or myc tag as appropriate. All constructs used in this study were stable in this assay (not shown).

4.2.2. Xbra is localised to chromatin during the cell cycle

To investigate the nuclear localisation of Xbra protein, RNA encoding HA tagged Xbra (XbraHA) was injected into one cell of the 8 cell stage embryo, and the protein was visualised by whole mount antibody staining between stages 8.0 and 9.0. At these stages endogenous Xbra is not yet expressed to a detectable level, so that visualised protein had to derive from the injected RNA. In initial experiments the XbraHA RNA was co-injected with fluorescein lysine dextran (Molecular Probes) to visualise the area of injection. In all cases examined the protein was located in the nucleus, irrespective of the area where Xbra was expressed (Fig. 4.6B, C and data not shown). This is unlike for the transcription factor myoD, where ectopically expressed protein is localised to the cytoplasm and enters nucleus only in areas where the endogenous gene is expressed (Rupp *et al.*, 1994). However, the nuclear localisation of ectopic Xbra is consistent with the fact that the protein can induce mesoderm when over expressed in the animal cap (Cunliffe and Smith, 1992).

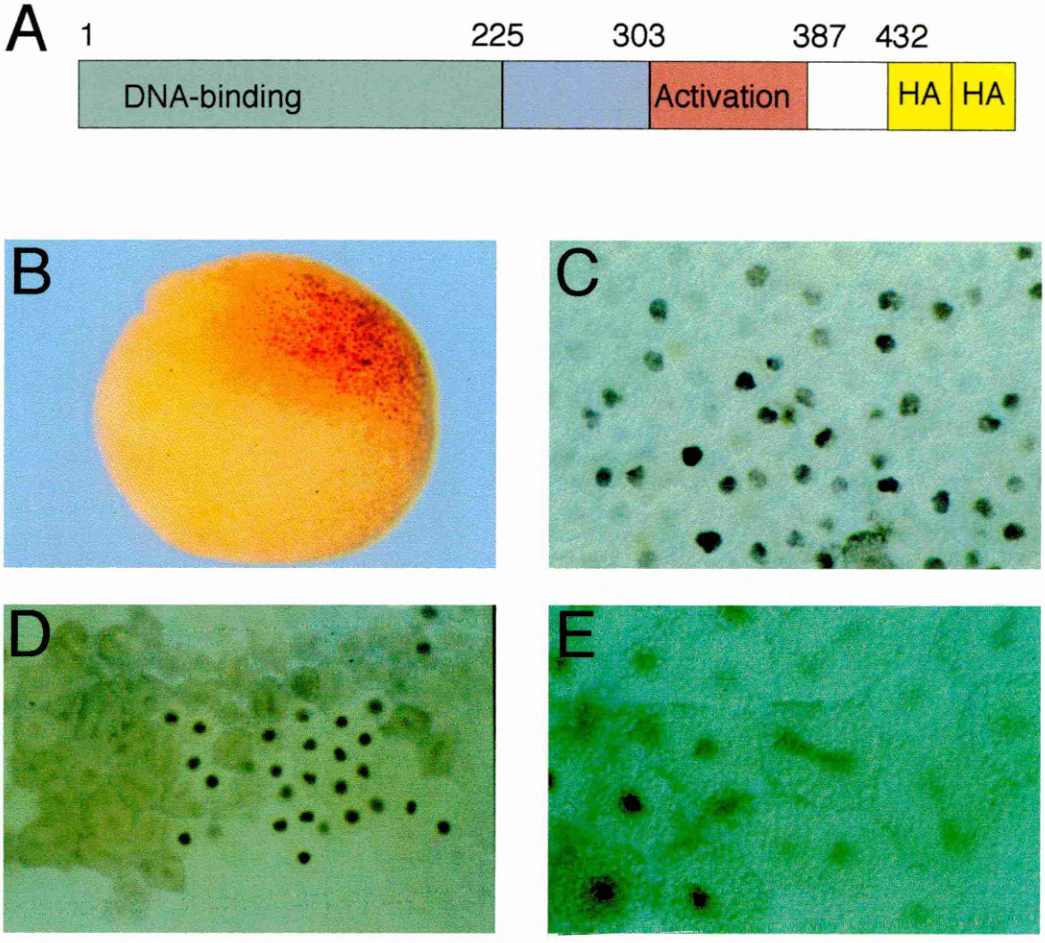


Figure 4.5. Cellular localisation of HA-tagged full-length Xbra protein. **A.** Schematic diagram of the injected construct. Xbra domain structure as in Fig. 4.3. Two HA tags were added to the c-terminua. **B-E.** Embryos were injected into one cell at the 8-cell stage. **B.** XbraHA was co-injected with fluorescein dextran lysate. XbraHA was localised by immuno-staining with a primary monoclonal antibody against the HA tag and a secondary αmouseIgG antibody conjugated with HRP (horse radish peroxidase). Detection was performed with DAB and NiCl. Fluorescein dextran lysate was visualised with αfluorescein Fab fragments fused to AP (alkaline phosphadase) and colour reaction with fast red. Nuclear DAB staining and staining by Fast Red co-localised in all regions of the embryo. **C.** Injected, non-dividing ells visualised with αHA antibody. **D.** Injected mitotic cells alongside interphase cells.; **E.** Higher magnification of an area in D. Localisation of Xbra HA in D, E was visualised with αXbra antibody (BF163) to achieve better resolution.

The XbraHA protein was detected with either a monoclonal antibody against the HA epitope (Boehringer Mannheim) or with a polyclonal rabbit antiserum raised against the full-length protein (FB163, Brenda Price and Jim Smith, unpublished) with identical results. Interestingly, most XbraHA protein stayed localised to the center of cells in during mitosis (Fig. 4.6D, E). Although the chromatin was not separately visualised in these experiments, staining is probably marking metaphase chromosomes. This is unusual, because most transcription factors, such as Oct-1, Oct-2, Ets-1, B-Myb, c-Fos, EF-1 and Bcl-6 are actively displaced from the chromatin during mitosis (Martinez-Balbas *et al.*, 1995). However, localisation to the chromatin during metaphase is also observed for the transcription activator AP-2 (Martinez-Balbas *et al.*, 1995).

4.2.3. Deletion analysis suggests a bipartite nuclear localisation signal

In order to identify the domains responsible for nuclear localisation of Xbra I investigated the localisation of several N-terminal and C-terminal deletion constructs (Fig. 4.6). The procedure was identical to that in 4.2.2. Although detection of the truncated proteins was also performed with monoclonal antibodies against the HA-epitope (antiHA, Boehringer Mannheim) and the myc-epitope (9E10, Santa Cruz Biotechnology Inc.), the results presented here came from experiments using the polyclonal rabbit antiserum FB163. The higher specificity of the latter made it possible to achieve a better resolution and in addition the procedure could be standardised for the use of N-terminal and C-terminal deletion constructs. However, the results with the monoclonal antibodies against the epitope tags were consistent with those shown in Fig. 4.6.

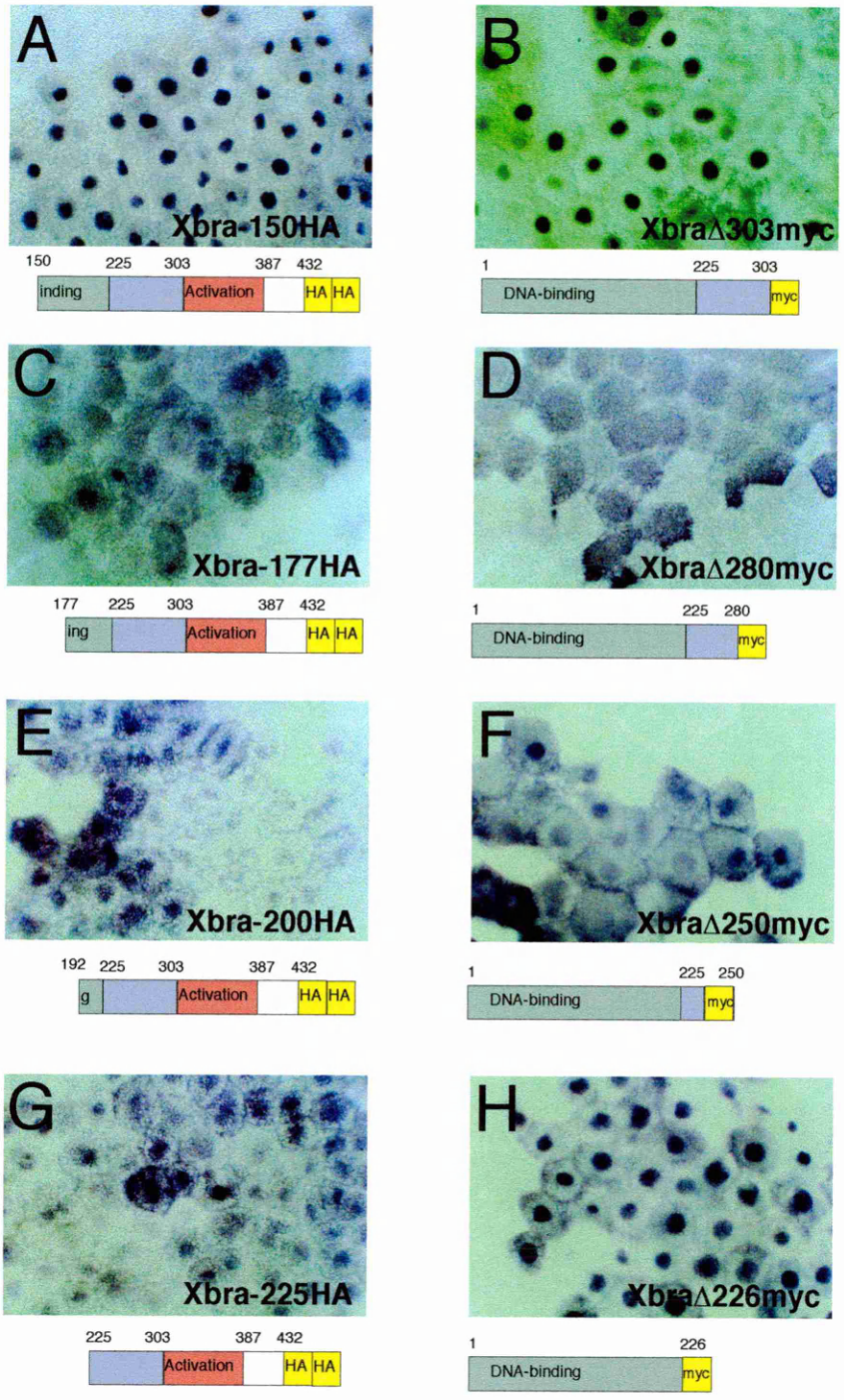


Figure 4.6. A-H. Cellular localisation of Xbra N-terminal and C-terminal deletion proteins. RNA encoding the Xbra deletion was injected into an animal hemisphere cell of the 8-cell embryo. Schematic view of protein encoded by the injected construct is shown below the picture of stained cells. Localisation of protein was visualised with αXbra antibody (BF163) at embryonic stage 8.5.

N-terminal deletions of up to 150 amino acids, or C-terminal deletion up to amino acid 303, did not have any effect on the nuclear localisation of the proteins (Fig. 4.6A, B). However, when the C-terminus was further deleted to amino acid 280, nuclear localisation was completely lost and the protein was equally distributed between nucleus and cytoplasm (Fig. 4.6D). Surprisingly, when the C-terminus of Xbra was further deleted to 250 amino acids, a larger proportion of the protein was again localised to the nuclei (Fig. 4.6F) and further deletion to amino acid 226 nearly completely restored nuclear staining (Fig. 4.6H). N-terminal deletion to amino acid 177, 200 and 225 resulted in cytoplasmic and nuclear staining, with more protein located in the nucleus (Fig. 4.6C, E, G). These results suggest that domains between amino acids 150 and 177 and amino acids 280 and 303 are both involved in conferring nuclear localisation of the Xbra protein. In addition they indicate that the region between amino

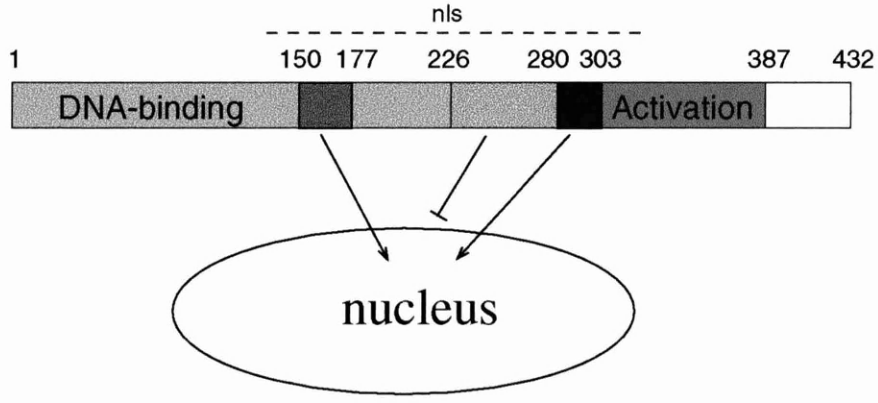


Fig. 4.7. Regions involved in nuclear localisation of Xbra, as suggested by the deletion study. Regions between amino acids 150-177 and 280-303 are acting positively, while the region between amino acid 226-280 appears to act negatively towards trans-location into the nucleus. nls: region responsible for nuclear localisation as suggested by Kispert *et al.* (1995)

acids 226 and 280 negatively affects nuclear localisation (see Fig. 4.7 for summary). However, more experiments are necessary to investigate whether these regions are necessary and sufficient for nuclear localisation.

4.2.4. Both components of a Xbra homo-dimer need DNA binding to dimerise on DNA

Crystallisation of the Xbra T-domain on a palindromic DNA binding site suggested that Xbra binds to this site as a homodimer (Muller and Herrmann, 1997). Binding of Brachyury as a homodimer to this site was also suggested by studies using human T protein, which showed that full-length T can form (albeit with low stability) heterodimeric complexes with truncated protein consisting only of the T-domain (Papapetrou *et al.*, 1997). These results contrasted with the initial Brachyury DNA binding study of Kispert and Hermann (1993), who did not see such interaction between full-length protein and T domain, and thus they incorrectly concluded that Brachyury binds the palindromic binding site as a monomer.

I repeated this experiment using full-length Xbra protein together with a C-terminally truncated form of Xbra, which lacks the activation domain but still contains the T-domain and the linker domain (XbraΔ303). The proteins were in vitro translated from RNA in a rabbit reticolysate extract (Promega). Both full-length protein ($Xbra^{fl}$ Fig 4.8A, lane 1) and XbraΔ303 (Fig. 4.8A, lane 6) bind the palindromic sequence, with XbraΔ303 migrating with a higher mobility than $Xbra^{fl}$. When both proteins are translated in the same extract (Fig. 4.8A, lanes 11 and 17), the corresponding bands to $Xbra^{fl}$ and XbraΔ303 are observed, but so is an intermediate band, strongly suggesting

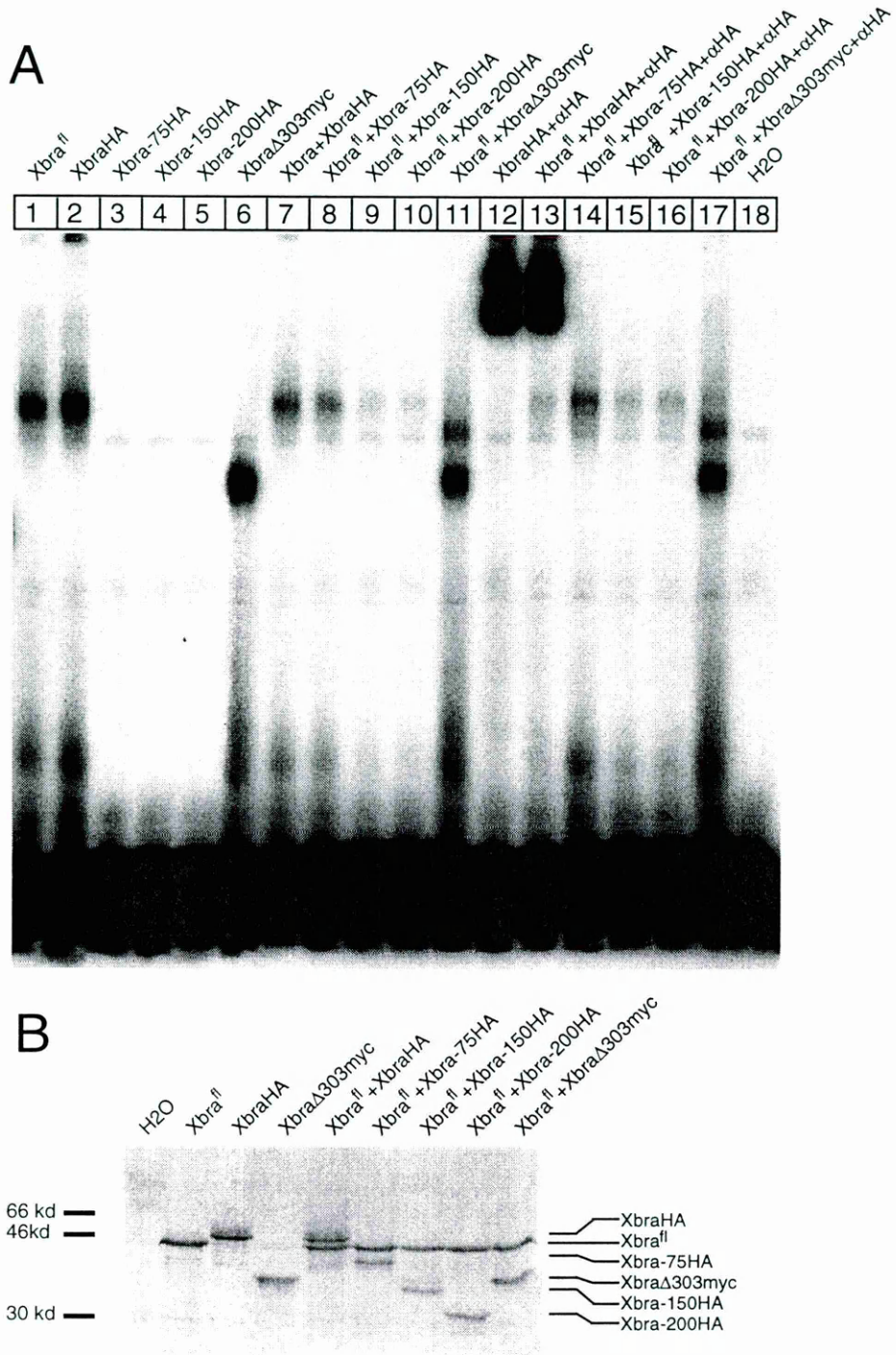


Fig. 4.8. A. EMSA of co-translated Xbra constructs analysing dimerisation on a palindromic Brachury binding site. See text for details. **B.** Immunoblot of the in vitro translated Xbra constructs used in A. Xbra construct were visualised with the αXbra polyclonal rabbit serum.

dimer-formation between these two forms of the protein. Interestingly, the homodimer of XbraΔ303 appears to have significantly stronger affinity for the palindromic sequence than the homodimer of the full-length protein, suggesting steric constraints which inhibit the full-length-protein from forming a dimer on this sequence. Addition of αHA antibody to extracts containing HA-tagged full-length protein strongly enhances the protein DNA complex formation (Fig. 4.8A lanes 12 and 13), possibly by facilitated dimer formation through the two antigen binding sites of the antibody.

The above section showed that dimers of full-length protein do form on DNA. To further address the question whether they can form without binding to DNA, I was interested to know whether full-length Xbra is able to interact with a N-terminal deletion that is not able to bind DNA. None of the N-terminal deletions used in this study (those lacking 75 or more amino acids) was able to bind the palindromic sequence by themselves (Fig. 4.8A, lanes 3-5). However, they are also unable to form a protein:DNA complex when co-translated with full-length Xbra protein (Fig. 4.8A, lanes 7-10). Supershift of the N-terminally truncated proteins confirmed that no complexes with the full-length protein on DNA had formed (Fig. 4.8A, lanes 12-16). Immunobloting of the extracts using the polyclonal αXbra antiserum (FB163) confirmed the integrity and equal production of the proteins (Fig. 4.8B)

Experiments of this sort could not be performed using a 'half site' of the palindrome, because under the *in vitro* conditions used here and in other studies (Kispert and Hermann, 1993, Casey *et al.*, 1998b), full-length Xbra protein does not consistently bind to a half site.

4.2.5. Co-immunoprecipitation shows no evidence for homo-dimerisation in solution

The above section showed that dimers between an N-terminally truncated Xbra protein and a full-length Xbra protein cannot form on a palindromic T-box binding site. However, they do not address the question of whether such dimers exist in solution.

In order to address this question, XbraHA constructs were co-translated with Xbra constructs lacking an HA epitope. The sizes of the proteins with and without an HA tag differed, when subjected to SDS PAGE, allowing one to distinguish them on

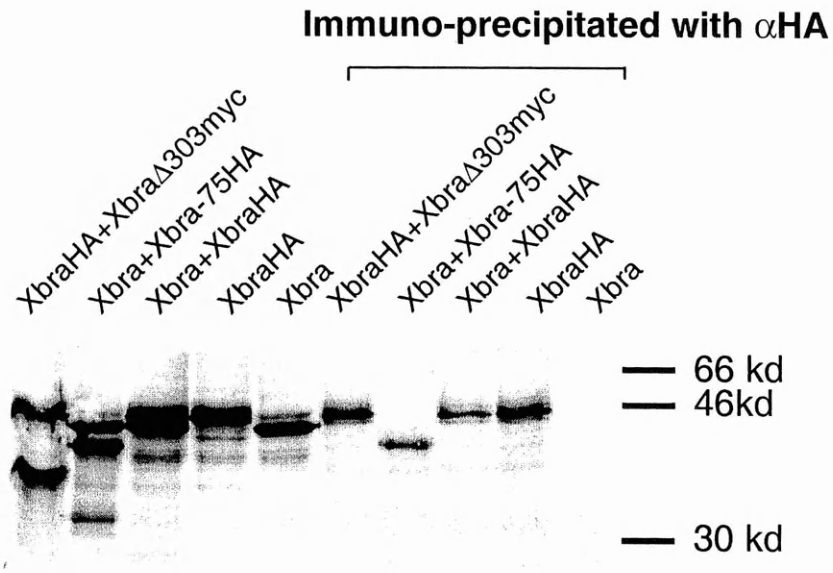


Fig. 4.9. Co-immunoprecipitation of Xbra constructs with and without HA-tag. The Proteins were co-translated from RNA in the same tube of rabbit reticulocyte extract in the presence of ^{35}S-methionine. The first five lanes were loaded with extract after in vitro translation. The last 5 lanes were loaded after the extract was immuno-precipitated with αHA antibody. The gel was exposed to X-ray film for 4 hours. Only the HA tagged proteins are present following immuno-precipitation, even after prolonged exposure of the film (not shown)

immuno-blots (Fig. 4.9). These extracts were then immunoprecipitated with αHA antibody (Boehringer Mannheim). If dimers between the constructs were able to form in solution, proteins without the HA tag should co-precipitate with the tagged constructs. However no such co-precipitation was observed (Fig. 4.9 and data not shown), arguing against the formation of dimers in solution.

4.2.6. The linker region of Xbra can be phosphorylated by MAP kinase

Examination of the protein sequences of *Xenopus*, mouse, chick and zebrafish Brachyury reveals several conserved putative consensus sites for phosphorylation by MAP kinase (PD[S/T]P or [S/T]P) (Fig. 4.10). In this study I decided to concentrate on amino acids 1 to 303. This region is of interest, because it can interact with full-length Xbra in a yeast two hybrid test (Frank Conlon, unpublished, see Fig. 4.2) and also contains the regions expected to mediate nuclear localisation of Xbra (see above and Kispert *et al.*, 1995a). In addition it is the largest fragment that could be efficiently expressed in E-coli (Richard Tyrell, unpublished observation). Within this region are three conserved putative MAP kinase phosphorylation sites (Fig. 4.10; MK1, MK2, MK3). Each of them is located in an interesting position.

MK1, which is located in the DNA binding domain, represents a perfect match for a MAP kinase site (PDSP) and is conserved in all vertebrate Brachyury homologues (Fig. 4.10). In addition it overlaps with the region involved in the dimerisation interface when the T-domain is crystallised on a palindromic recognition sequence (Muller and Herrmann, 1997).

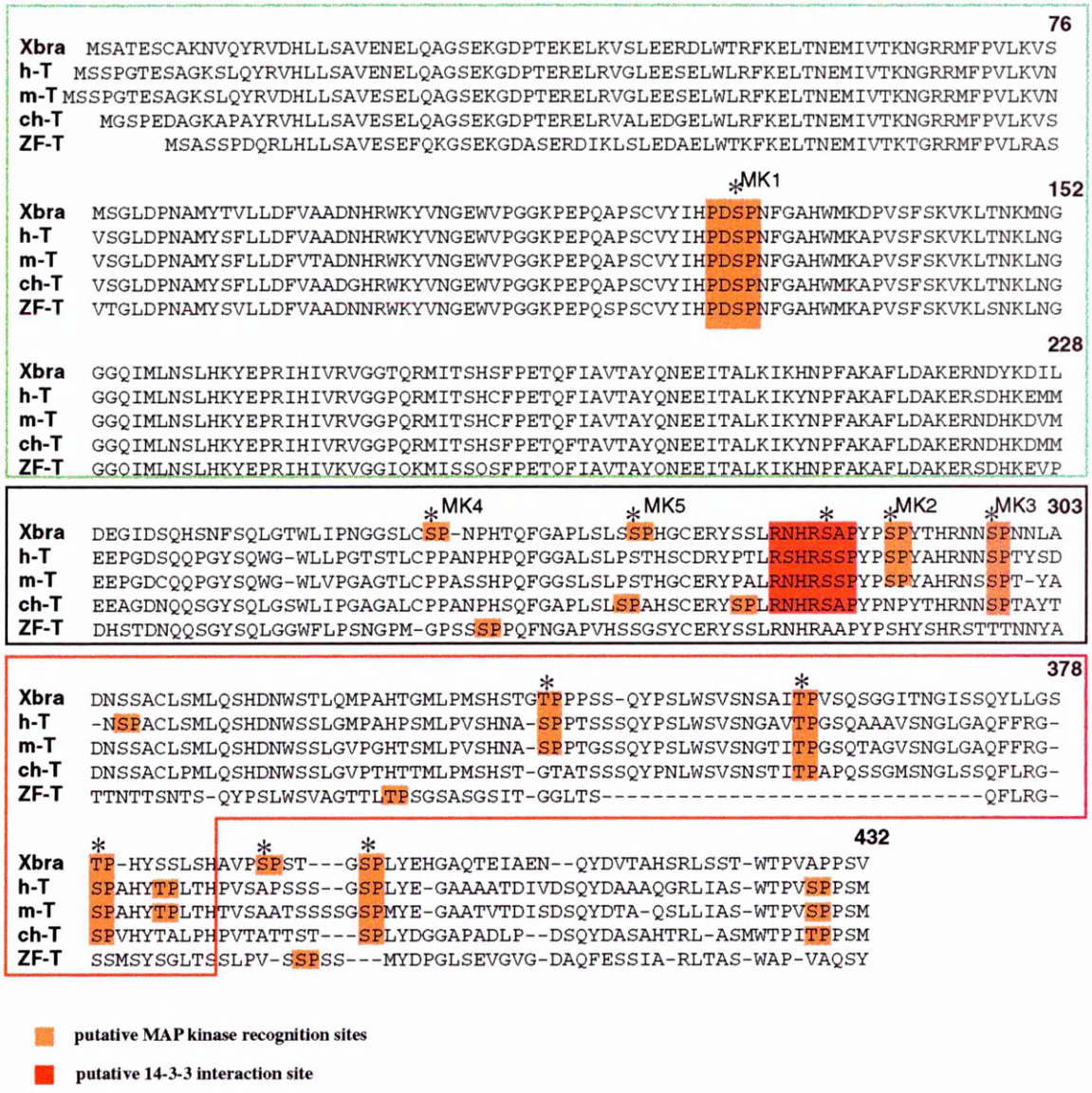


Fig. 4.10. Protein sequence of the Xbra protein aligned with protein sequences from human (h-T), mouse (m-T), chicken (ch-T) and zebrafish (ZF-T) homologues. Positions are only given for the Xbra protein. Coloured outlining marks the Xbra protein domains (green: DNA binding domain, blue: linker domain, red: activation domain). *:putative phosphorylation sites. Putative MAP kinase sites are boxed in orange, the putative 14-3-3 interaction site is boxed in red.

MK2 and MK3 are located at the C-terminal end of the linker domain (Fig 4.10), which might be involved in mediating part of the nuclear localisation signal (Chapter 4.2.3). They are also in close proximity to a putative 14-3-3 interaction site (Fig. 4.10, RX[Ar/H][+]pSXP; where Ar = aromatic, + = basic residue, pS = phosphoserine; Yaffe *et al.*, 1997). The Xbra protein contains two additional MAP kinase consensus sites in the linker region (Fig. 4.10, MK4, MK5), but these sites are not conserved in Brachyury proteins from other vertebrates.

To investigate whether MAP kinase is able to phosphorylate theses sites *in vitro* I performed phosphorylation assays on bacterially expressed His-tagged XbraΔ303 and His tagged XbraΔ228 (Fig. 4.11). These experiments were carried out in collaboration with Richard Tyrell (Division of Protein Structure, NIMR, London). In an initial experiment XbraΔ303 was incubated with purified MAP kinase (New England Biolabs) in the presence of γ^{32}P-ATP. Aliquotes of the reaction were stopped after different time points ranging from 1 minute to 60 minutes (Fig. 4.11A). Under these conditions phosphorylation of XbraΔ303 by MAP kinase occurs in as little as 5 minutes and the reaction reaches its plateau between 30 minutes and 60 minutes. Thus for the further experiments we chose the time points 15 minutes and 30 minutes.

We asked next whether XbraΔ228, which contains the T-box but not the linker region, can be phosphorylated by MAP kinase under these experimental conditions. However, to our initial surprise this was not the case (Fig. 4.11B). After 30 minutes incubation when phosphorylation of XbraΔ303 was clearly detectable, no incorporation of γ^{32}P was observed in XbraΔ228. Even after prolonged incubation of MK-assays for 90

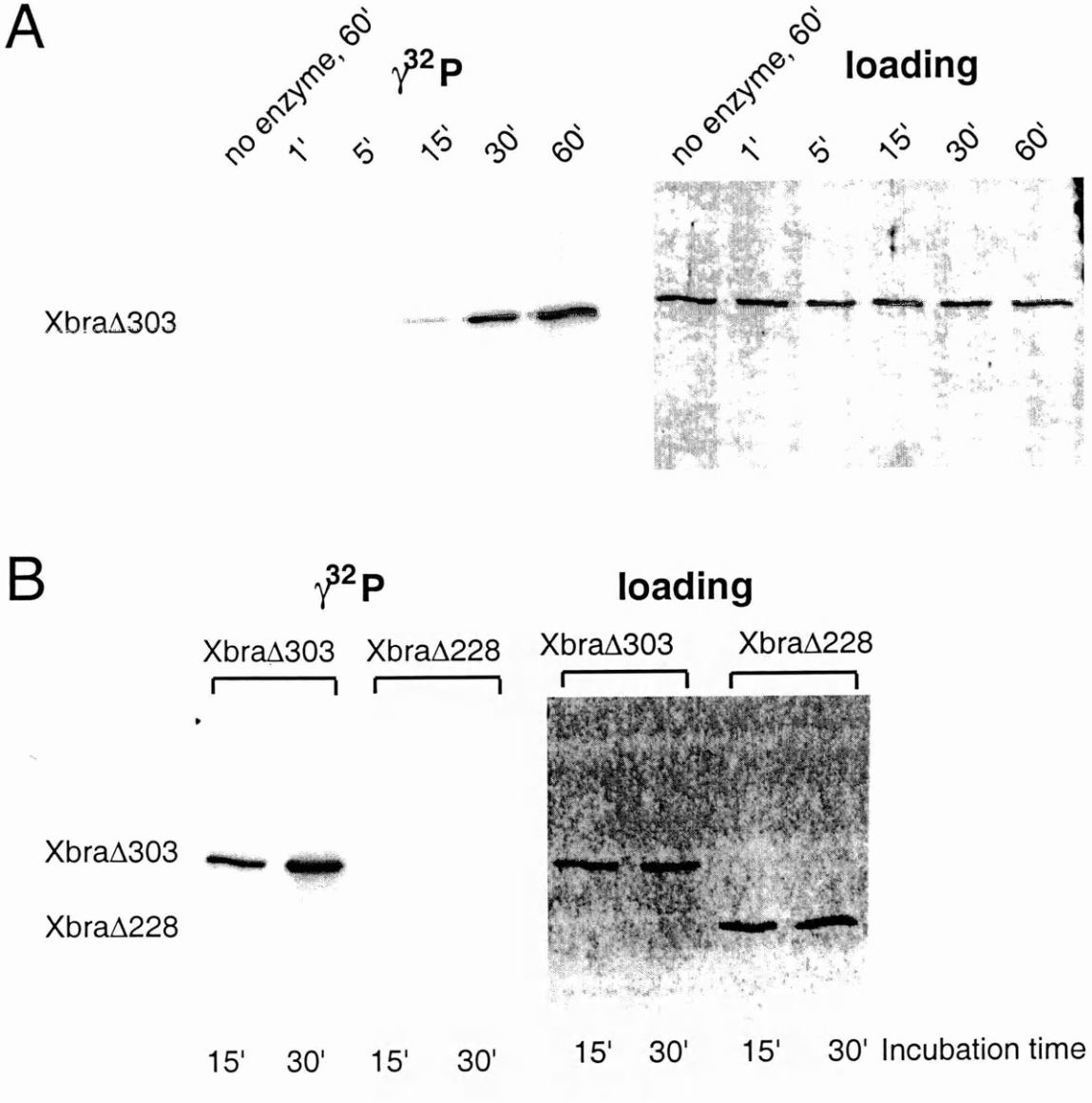


Fig.4.11. ***In vitro*** **MAP kinase assays with affinity purified Xbra protein constructs in presents of γ^{32}P-ATP. A.** Left panel: Time course of XbraΔ303 incubated with MAP kinase and γ^{32}P-ATP, separated on an acryl amide gel and exposed to X-ray film. Incorporated γ^{32}P increases between 1 minute and 60 minutes; right panel: Same gel stained with Bromphenol blue confirms approximately equal loading. **B.** Incorporation of γ^{32}P in presence of MAP kinase occurs in XbraΔ303, but not in the shorter XbraΔ228

minutes and long exposure of the film no indication of phosphorylation of the 228 amino acid T-domain was observed (not shown). The most likely explanation is that although this fragment contains a conserved PDSP MAP kinase consensus recognition site, this site is not accessible to the MAP kinase enzyme. Thus the observed phosphorylation of the XbraΔ303 fragment is likely to occur in the linker region of Xbra, perhaps at the conserved MK2 or MK3 sites.

In a preliminary investigation to ask whether the MK2 or the MK3 consensus sites in the linker region of Xbra are phosphorylated by MAP kinase, I created XbraΔ303 constructs with point mutations in the MK2 site (XbraΔ303MK2^{mut}) or the MK3 site (XbraΔ303MK3^{mut}) by replacing the serine residues of the PS sites to an alanine (PS→AS). However, incubation of bacterially expressed proteins from these constructs with MAP kinase in presence of γ^{32}P-ATP did not show any significant

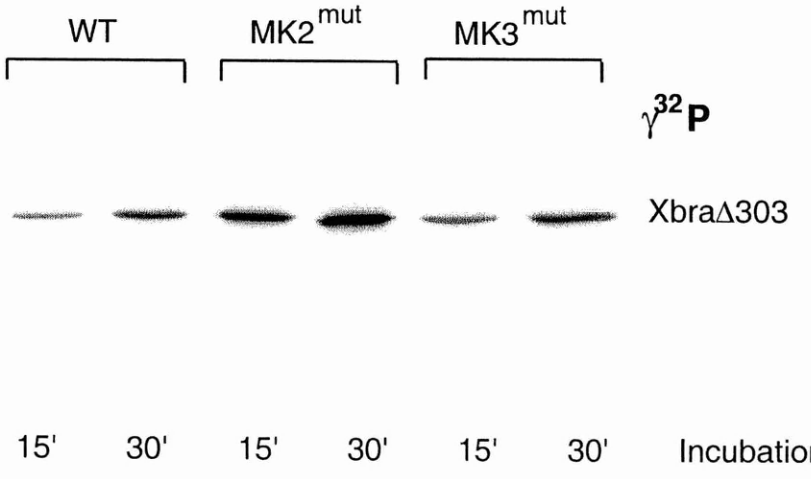


Fig. 4.12. In vitro MAP kinase assay in presence of γ^{32}P-ATP with wildtype XbraΔ303 protein or with XbraΔ303 protein with point mutations in individual MAP kinase sites. No significant difference in incorporation of γ^{32}P is observed.

changes in the phosphorylation characteristics of these proteins (Fig. 4.12). Constructs with point mutations in the other two PS consensus sites (MK4 and MK5) or a construct with a double mutation in MK2 and MK3 have not been tested yet in this assay.

The above results do not necessarily indicate that MK2 and MK3 are not required for phosphorylation by MAP kinase. It could merely mean that several or all of the MAP kinase sites in the linker domain are phosphorylated in vitro, and that it will be necessary to investigate the functional significance of the sites in the embryo. To this end I performed already preliminary experiments in which a full-length Xbra protein carrying a mutation in the MK2 site (XbraMK2^{mut}) was over-expressed in the embryo. These experiments indicated animal hemisphere tissue from such embryos has different characteristics from the mesoderm induced by wildtype Xbra. In particular many of the animal pole explants which over-expressed XbraMK2^{mut} formed patches of pigment, while none of the animal pole explants over-expressing wildtype Xbra showed this phenotype (data not shown). However, more detailed experiments, including analysis by molecular markers is necessary before any conclusions can be drawn.

Future experiments should also address the phosphorylation characteristics of the wildtype and mutated proteins in vivo using pulse chase experiments of animal hemisphere cells incubated with FGF. Preliminary experiments, which investigated phosphorylation of the endogenous gene by incubating marginal zone cells with γ^{32}P-ATP and immunoprecipitation with αXbra antiserum suggested that phosphorylation of Xbra does occur in vivo (Brenda Price and Jim Smith, unpublished observation).

4.3. Discussion and Future Experiments

In this chapter I have began to address different functional aspects of the Xbra protein. The search for the nuclear localisation signal has identified two regions likely to be involved in localising the protein to the nucleus. Concerning the question of whether Xbra acts as a monomer or a homodimer, I did not find any evidence for Xbra homodimers in solution. Full-length Xbra does bind the palindromic recognition site as a dimer, but for such a complex to form both components of the dimer need DNA binding activity. Additionally I have shown that Xbra protein can be phosphorylated by MAP kinase *in vitro*, and that this phosphorylation occurs in the linker region, but not in the DNA binding domain. Together these results present the basis for a detailed characterisation of the Xbra protein, which might not only be important for the understanding of Brachyury itself, but for the whole family of T-box factors. Future experiments for further investigations are suggested.

4.3.1. Does Brachyury utilise a novel mechanism for nuclear translocation?

Xbra domains involved in nuclear localisation

This study suggests two distinct regions in the Xbra protein that could be involved in facilitating its nuclear localisation, and one region that appears to have an negative influence on nuclear localisation (Fig 4.7). The results agree with the study of Kispert *et al.* (1995), who inferred complex nuclear regulation signals between the region of amino acid 138 and 320 of the mouse Brachyury protein. Interestingly, in two of their deletion (T1-300 and T156-400) they observe primary localisation in the nucleus, but part of the protein in the cytoplasm. These deletions are only 3 amino acids shorter on the C-

terminus and only 6 amino acids shorter on the N-terminus than the longest deletions that are still completely localised to the nucleus in the present study. Together these results are consistent with the involvement of the two identified regions (150-177 and 280-303) to be involved in nuclear localisation. However, to conclusively confirm this, additional experiments have to be conducted:

(i) It needs to be shown that internal deletion of one or both the regions result in cytoplasmic localisation of the Xbra protein
(ii) Fusion of one or both the regions should be able to confer nuclear localisation when fused to an otherwise cytoplasmic protein, such as beta-galactosidase.

What is the mechanism of nuclear localisation of the Xbra protein?

None of the regions in Xbra, thought to be involved in nuclear localisation have any obvious homology to known nuclear localisation signals (reviewed in Jans and Hubner, 1996, Gorlich, 1997). Two pathways mediating nuclear import are known and have been characterised. The first pathway involves the recognition of a short stretch of basic amino acids by the NLS receptor importin. (Gorlich *et al.*, 1994). The other pathway functions via a different nuclear transport receptor, transportin (Pollard *et al.*, 1996), which recognises the M9 domain of the hnRNPA proteins (Siomi and Dreyfuss, 1995; Weighardt *et al.*, 1995). These two pathways are independent and do not compete with each other.

Both nuclear transport receptors, transportin and importin are limited in the cell. To investigate of whether nuclear localisation of Brachyury is mediated by either of

the pathway competition experiments could be performed. Such experiments make use of the digitonin-permeabilised cell import assay (Adam et al. 1990), where the outer cell membrane is permeabilised to allow transport of macro-molecules, but the nuclear membrane is kept intact. If such cells are incubated with a transport solution containing a cytosolic extract mixed with a purified protein, this protein becomes localised to the nucleus if it has a nuclear localisation signal. If transport of the protein is mediated by importin then addition of a SV40 large T NLS peptide (PKKKRKV) but not the inactive reverse sequence (SLN) will compete with the transport into the nucleus (Adams *et al.*, 1990). If the transport of the protein is mediated by transportin then a peptide containing the functional M9 domain but not the mutated M9 domain (Michael *et al.*, 1995) will compete with the transport (Pollard *et al.*, 1996). To perform this experiment for Brachyury, bacterially expressed and purified XbraΔ303 would be used. SV40 large T NLS and SLN are commercially available. Peptide containing the functional or mutated M9 domain would be best obtained from the laboratory of Gideon Dreyfuss (Department of Biochemistry and Biophysics, University of Pennsylvania, USA).

The Xbra region between 280 and 303 contains a conserved putative interaction site for 14-3-3 proteins and two conserved putative MAP kinase consensus sites (MK4 and MK5). Interestingly, members of 14-3-3 family have recently been implicated in the nuclear export machinery together with the nuclear export protein Crm1 (Lopez-Girona *et al.*, 1999). This pathway is responsible for the cytoplasmic localisation of the mitotic inducer Cdc25 and the protein kinase Ualpha during interphase (Kumagai and Dunphy, 1999, Zhang *et al.*, 1999). Nuclear export involving 14-3-3 of these proteins is not achieved by cytoplasmic retention, but the export machinery has to be actively maintained (Kumagai and Dunphy, 1999). Thus 14-3-3 has

to shuttle between nucleus and cytoplasm. Members of the 14-3-3 family are thought to work as molecular scaffolds or chaperons, by promoting the contact between interacting proteins (Aitken, 1996). This means they can be employed by several different pathways, depending on the proteins whose interaction they promote. A function in the nuclear import of Xbra can thus not be excluded and should be investigated. 14-3-3 binds to its recognition site only when the included serine is phosphorylated. Thus a point-mutation changing the serine to an alanine should disrupt any interaction and should affect nuclear localisation in case 14-3-3 is involved.

Future experiments also should investigate the nuclear localisation of constructs with point mutations in the putative MAP kinase sites in this region (MK2, MK3). Disruption of the MK2 site alone in the background of full-length Xbra (XbraMK2^{mut}), does not have any effect on nuclear localisation (data not shown). I have also created already the construct with a mutation in the MK3 site, however I have not yet tested the effect on its nuclear localisation. Interestingly, this site is deleted in the mouse Brachyury C-terminal deletion (T1-300; Kispert *et al.*, 1995a), which shows partial cytoplasmic localisation, however, is present in the XbraΔ303 which is located entirely in the nucleus (this study).

4.3.2. Localisation of Xbra to chromatin during metaphase

Investigation of the nuclear localisation of Xbra protein suggests that it stays localised to the chromatin during mitosis. While most transcriptions factors have unspecific affinity to the chromatin during interphase, they are actively displaced from the metaphase chromosomes (Martinez-Balbas *et al.*, 1995). This is not due to a simple

dilution effect, because of the break down of the nuclear membrane, because when the nuclear membrane is damaged by Nonidet P-40 (NP-40) during interphase, a large proportion of those same transcription factors stays attached to the chromatin (Martinez-Balbas *et al.*, 1995). It was suggested that the dispersal of transcription factors coincides with the process of chromatin condensation, and the resulting histone deacetylation (Chahal *et al.*, 1980, Turner, 1989, Wade *et al.*, 1997). Interetingly DNaseI hypersensitive site keep intact during this displacement for several promoters studied (Kuo *et al.*, 1982; Martinez-Balbas *et al.*, 1995), which probably helps the reassembling of the transcription factors on the decondensing chromatin.

The bulk dispersal from mitotic chromosomes appears to be characteristic for many transcription factors, but Xbra would not be the only exception. The sequence specific transcription activator AP-2 and the serum response factor $p67^{SRF}$ show association with the condensed metaphase chromatin (Martinez-Balbas *et al.*, 1995, Gauthier-Rouviere *et al.*, 1991). It would be interesting to investigate the mechanism by which these transcription factors escape the displacement and whether it has any functional significance.

4.3.3. Dimerisation of Xbra on DNA

My results have shown that a full-length Xbra and a C-terminal deletion of Xbra containing the linker region (XbraΔ303) can bind to a palindromic recognition site as a heterodimer. Interestingly, such dimer formation was not found between T-domain alone (T1-226) and the full-length protein in a study by Kispert and Hermann, 1993. A later study however, using a truncated human T domain (hT1-233) found dimer

formation between the truncated and full-length Brachyury, although dimer interaction of the truncated protein was rather low under their conditions with considerable amount of protein present as a monomer (Papapetrou *et al.*, 1997).

The results presented here suggest that XbraΔ303 binds the palindromic sequence more effectively than the full-length protein and heterodimers between these two reflect this distribution (see Fig. 4.8, lanes 11 and 17). I did not investigate the dimerisation properties of the T-domain alone (XbraΔ226) or in combination with the full-length protein. However, if it would remain correct that XbradΔ303 forms more stable dimers than the T-domain alone, than this would indicate that it contains regions that enhance the weak dimer interface observed in the crystallographic structure of the domain bound to DNA, possibly changing the mode of dimer formation. My results also suggest that Xbra is not normally present as a dimer in solution. However, it is still possible that dimer-formation of Xbra is facilitated by an unrelated protein present in the embryo, but not in the retico lysate used for in vitro translation.

Addition of antibody strongly enhances binding of the full length protein to the DNA (this study, Kispert and Hermann, 1993). This is likely due to stabilisation of the dimer on DNA through the two antigen binding sites of the antibody. It is possible that endogenous adapter proteins play a similar role in the embryo. All target genes of Brachyury studied so far contained only 'half sites' of the palindromic binding site in their promoter. This indicates that Brachyury binds to these promoter as a monomer, or in complex with a different protein (Casey *et al.*, 1998a; Tada *et al.*, 1998). The only palindromic binding site identified so far is in the ascidian homologue of *Brachyury* (*As-T*) itself (Takahashi *et al.*, 1999). *As-T* is expressed only in the notochord (Yasuo

DNA (Muller and Herrmann, 1997). No phosphorylation has been observed in the structure, thus it would be likely that it would interfere with dimer formation, rather than stabilising the protein.

However, we could show that the linker domain of the Xbra domain is phosphorylated by MAP kinase *in vitro*. The two conserved putative MAP kinase sites within the linker domain are in on of the region that is thought to be important for nuclear localisation. In addition they are located very near to the putative 14-3-3 interaction site. Beside its possible role in nuclear localisation and facilitation of dimer formation 14-3-3 is also implicated in the MAP kinase pathway. It has been shown to positively regulate this pathway by binding to Raf and facilitating the complex formation with down stream signalling molecules and as effector of serine phosphorylation (Chang, Rubin, 1997, Thorson 98, Tzivion, 98). However it is not clear whether this influence would reach to the transcription factor level.

The importance, specifically of the MK2 and MK3 sites will be addressed in future experiments in the embryo by overexpression of Xbra constructs with individual and double mutations in these sites. Two different mutations for each site will be applied. A substitution from a serine to an alanine will make the sites inaccessible for phosphorylation and thus would represent a constitutively unphosphorylated form. In contrast a substitution of the serine by an aspartic acid will mimicking the effect of serine phosphorylation and thus make the site constitutively active (Casanova *et al.*, 1990; Hurley *et al.*, 1990). Such constitutively active mutation of the serines 362 and 374 for example greatly enhanced the stability of C-fos in cell culture (Okazaki and Sagata, 1995).

and Satoh, 1994). Notochord specific targets of *Brachyury* have not been identified yet in higher vertebrates, nor has the promoter element been found that is responsible for expression of *Brachyury* in the notochord in these species. Thus it will be interesting to see whether a palindromic binding site is part of such an element.

Intriguingly, a *Xenopus 14-3-3* homologue has been identified, that is only expressed in the notochord (Frank Conlon, Betty Baker, Jim Smith, unpublished observation). Keeping in mind that 14-3-3 proteins acts as a dimer (Liu *et al.*, 1995; Xiao *et al.*, 1995), and Xbra has a putative binding site for 14-3-3 (this study), it is possible that 14-3-3 could facilitate dimerisation specifically in this tissue. However, although the *Xenopus* 14-3-3 homologue has been identified in a two-hybrid screen for Xbra interacting factors, proof of interaction of these two proteins in *Xenopus* embryos has been difficult (Frank Conlon, Betty Baker, Jim Smith, unpublished observation).

4.3.4. Is Xbra protein regulated by the MAP kinase pathway?

Comparison the Xbra protein sequence with Brachyury homologues form other higher vertebrates has revealed several putative consensus sites for MAP kinase that are conserved in most of the species. The only exception is ZF-T, the zebrafish homologue, in which none the consensus sites are conserved in the C-terminal half of the protein. In collaboration with Richard Tyrell (Department of Protein structure, NIMR) I could show that the consensus site in the N-terminal half of the protein is not phosphorylated by MAP kinase *in vitro*. This was initially surprising, because it is a perfect match for the consensus and conserved in all species. However, the exact same peptide was shown to be involved in formation of the dimer-interphase when the T-domain is bound to

Future experiments should also address the phosphorylation characteristics of the wildtype and mutated proteins in vivo using 'pulse chase' experiments of animal hemisphere cells incubated with FGF and γ^{32}P-ATP. Preliminary experiments, which investigated phosphorylation of the endogenous gene by incubating marginal zone cells incubated with γ^{32}P-ATP followed by immuno-precipitation with αXbra antiserum suggested that phosphorylation of Xbra does occur in vivo (Brenda Price and Jim Smith, unpublished observation). If phosphorylation changes the mobility of Xbra then the constructs with the mutated sites should be clearly distinguishable in motility from the wildtype form after treatment with FGF. Thus preliminary experiments could be done without the need of performing highly radioactive 'pulse chase' experiments.

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